The development of gene therapy for recessive dystrophic epidermolysis bullosa

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Awarding institution:
King's College London

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The development of gene therapy for recessive dystrophic epidermolysis bullosa

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This thesis is presented for the degree

Of University of London

2016

DECLARATION

 The work presented here is my own and all experiments, except where acknowledged in the text, were performed by myself.

Signature: …….

Date: …….

Relevant publications

1. Bone marrow transplantation in epidermolysis bullosa. **Abdul-Wahab A**, Petrof G, McGrath JA. 2012. *Immunotherapy* 12, 1859-67.

2. Cell therapy in dermatology. Petrof G, **Abdul-Wahab A**, McGrath JA. 2014. *Cold Spring Harb Perspect Med* 6, pii: a015156.

3. Lentiviral engineered fibroblasts expressing codon optimized COL7A1 restore anchoring fibrils in RDEB. Georgiadis C, Syed F, Petrova A, **Abdul-Wahab A**, Lwin SM, Farzaneh F, Chan L, Ghani S, Fleck RA, Glover L, McMillan JR, Chen M, Thrasher AJ, McGrath JA, Di WL, Qasim W. J Invest Dermatol. 2015 Sep 22. doi: 10.1038/jid.2015.364.

4. Phase I study protocol for ex-vivo lentiviral gene therapy for the inherited skin disease, Netherton Syndrome. Di WL, Mellerio JE, Bernadis C, Harper J, **Abdul-Wahab A**, Ghani S, Martinez-Queipo M, Hara H, McNicol AM, McGrath J, Thrasher AJ, Qasim W. Human Gen Ther Clin Devel 2013 : Oct 18.

5. Potential of systemic allogeneic mesenchymal stromal cell therapy for children with recessive dystrophic epidermolysis bullosa. Petrof G, Lwin SM, Martinez-Queipo M, **Abdul-Wahab A**, Tso S, Mellerio JE, Slaper-Cortenbach I, Boelens JJ, Tolar J, Veys P, Ofuya M, Peacock JL, Martinez AE, McGrath JA. J Invest Dermatol. 2015 Apr 23. doi: 10.1038/jid.2015.158

Abbreviations

AAV	Adeno-associated viral vector
AF	Alexa fluor
ATMP	Advanced therapy medicinal product
BEBSS	Birmingham Epidermolysis Bullosa Severity Score
BM	Bone marrow
BMSC	Bone marrow stem cells
BMT	Bone marrow transplant
BMZ	Basement membrane zone
BSA	Bovine serum albumin
CB	Cord blood
CCMO	Central Committee on Research Involving Human Subjects
CHMP	Committee for Medicinal Products for Human Use
CI	Confidence interval
CRF	Clinical Research Facilities
CRP	C-reactive protein
CXCR-4	Chemokine receptor type 4
DAMP	Damage-associated molecular pattern
DAPI	4', 6-diamidino-2-phenylindole
DDEB	Dominant dystrophic epidermolysis bullosa
DEB	Dystrophic epidermolysis bullosa
DEJ	Dermo-epidermal junction
DEXA	Dual-energy X-ray absorptiometry

DIF	Direct immunofluorescence
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
EB	Epidermolysis bullosa
EBOS	EB Oropharyngeal Severity
EBS	Epidermolysis bullosa simplex
EC	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunoabsorbent assay
EMLA	Eutectic Mixture of Local Anaesthetics
EpSC	Epidermal Stem Cell
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
FISH	Fluorescence in situ hybridisation
FN	Fibronectin
FSH	Follicle-stimulating hormone
GCSF	Granulocyte-colony stimulating factor
GLP	Good laboratory practice
GS	Glycine substitution
GSS	Global severity score

HB-EGF	Heparin-Binding EGF-like Growth Factor
HLA	Human leukocyte antigen
HMGB-1	High-mobility group box 1
HPC	Haematopoetic progenitor cells
IC	Intracellular
IIF	Indirect immunofluorescence
IL	Interleukin
iscorEB	Instrument for Scoring Clinical Outcome of Research for EB
ITT	Intention to treat
JEB	Junctional epidermolysis bullosa
LD	Lamina densa
LH	Luteinising hormone
LL	Lamina lucida
LTR	Long terminal repeats
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare Products Regulatory Agency
MOI	Multiplicity of Infection
MoMLV	Moloney murine leukaemia virus
MSC	Mesenchymal stromal (stem) cells
MUSE	Multilineage-differentiating stress-enduring
NC	Non collagenous
NHS	Normal human skin
NS	Not significant

OCT	Optimal Cutting Temperature compound
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR-α	Platelet-derived growth factor receptor alpha
PM	Plasma membrane
PTC	Premature termination codon
qRT-PCR	quantitative real-time reverse transcription PCR
RDEB	Recessive dystrophic epidermolysis bullosa
RNA	Ribonucleic acid
RPM	Revolutions per minute
SCC	Squamous cell carcinoma
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFFV	Spleen focus forming envelope virus
SLAM	Signalling lymphocyte activating molecule
SOP	Standard Operating Procedure
TBSA	Total body surface area
TEAE	Treatment-emergent adverse event
TEM	Transmission electron microscopy
TGFβ-1	Transforming growth factor beta 1
TNF-α	Tumour necrosis factor alpha
ZFN	Zinc finger nucleases

Acknowledgements

I would first and foremost like to thank my supervisor Professor John McGrath. His approach, drive and determination to deliver clinical trials for patients with Epidermolysis Bullosa has been awe inspiring. His unswerving motivation, work ethic and attention to detail will stay with me throughout my career. With his support, I very quickly settled into life as a clinical researcher, and very soon started to feel like a valued member of the team not only within the group but within the EB family worldwide. I would also like to thank my second supervisor Professor Jemima Mellerio to whom I am indebted for initially inspiring me to consider undertaking a research project with the Genetic Skin Disease Group, and whose constant guidance throughout my project as well as my clinical career has been invaluable. Within our group I would also like to particularly acknowledge Gabriela Petrof for all her support with the clinical trial work and development. I have yet to see anything phase her, and truly appreciate her calm reassurance during all the research stresses. I would also like to acknowledge Rashida Praminik for guiding me through the laboratory methods and protocols but also for teaching me the lesser known critical issues of keeping lab books pristine, the value of a label maker, and if it doesn't work just try and try again! In addition, I would like to acknowledge Magdalena Martinez-Queipo, Laura Proudfoot, and all the other members of the research teams on the 9th floor. Celine Pourreyon at Dundee was invaluable with helpful Western blot advice and support with keratinocyte culture. Due to the long processes involved in gene therapy clinical trials I have now handed over the baton to both Su Lwin and Ellie Rashid and feel so happy that the trials are in their extremely safe and capable hands. I would also like to thank the EB Clinical Nurse Specialists funded by DebRA as well as the staff at St John's Institute of Dermatology for their contribution to recruitment and support of the EBGen project. A special thanks to all the staff working at the Robin Eady National Epidermolysis

Bullosa Diagnostic Lab headed by Liu Lu for all their work on the mutation analysis and to James McMillan for the assistance with the immunofluorescence data.

I would like to thank Professor Alain Hovnanian at INSERM in Paris for appointing me to the GENEGRAFT project. I would also like to thank Matthias Titeux, Patricia Joseph Mathieu, Klaus Kuhlcke Fernando Larcher and Marcelo Del Rio who truly become my GENEFGRAFT surrogate family and to whom I wish all the best for continuing the project, alongside Su Lwin. I would also like to thank Waseem Qasim and Adrian Thrasher at the Institute of Child Health for the collaboration on the development of the LENTICOL project. In particular, I would like to acknowledge Christos Georgiadis, Syed Farhuttallah, Anastasia Petrova and Wei-Li Di with whom I have worked on the pre-clinical data. I would especially like to acknowledge Syed Farhuttallah for guiding me with the work on the migration assays and FACS analysis.

I would like to also thank my family and my husband Farid for their patience and support, and in particular to my nanny Nikoletta Elek for caring so well for my children Aida and Lina during the long, dark days of failed experiments and the more enjoyable days away from home in Paris and Modena.

Finally, I would like to thank all the patients and families with RDEB to whom this is dedicated. Life with RDEB is painful beyond most people's understanding, and yet their support and enthusiasm for the projects has been phenomenal. Thank you.

Abstract

Epidermolysis bullosa (EB) is a group of inherited mechanobullous disorders characterised by trauma induced blistering. One of the most severe subtypes is recessive dystrophic epidermolysis bullosa (RDEB). RDEB is due to loss of function mutations in the gene encoding type VII collagen (*COL7A1*), one of the main constituents of anchoring fibrils anchoring the basement membrane to the underlying dermis. There is no cure for this devastating condition although promising pre-clinical studies for strategies using genetic correction, protein replacement, cell therapy or drug therapies are underway. Reconstitution of *COL7A1* expression in both keratinocyte and fibroblast cell populations has been demonstrated using ex vivo gene therapy and hypothesised to lead to new anchoring fibril formation and amelioration of disease phenotype. Feasibility of this approach had been demonstrated in pre-clinical studies using a retroviral vector, and this work details the development of a phase 1 clinical trial to graft an autologous gene corrected skin equivalent graft. Detailed analysis of a cohort of adult patients with RDEB was performed in order to identify suitable trial candidates. In addition, an alternative strategy using a lentiviral vector encoding codon-optimised *COL7A1* developed in order to transduce fibroblasts to be administered intradermally was developed. Expression and secretion of full-length de novo C7 was confirmed, with transduced cells exhibiting increased levels of protein expression despite only modest transduction efficiencies. This work details the journey and obstacles encountered in developing gene therapy clinical trials for RDEB, both through the development of a phase 1 study for an autologous gene corrected skin equivalent graft as well as a phase I study of intradermal autologous gene corrected fibroblasts.

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Preface

My MD research period started in January 2012 and was completed in January 2015 and details only a segment of a long and challenging journey in the development of two phase I clinical trials of ex vivo gene therapy for RDEB. My three main goals were to

- Characterise a UK patient cohort for GENEGRAFT clinical trial
- Develop a clinical trial of autologous gene corrected fibroblasts for intradermal administration in adult patients with RDEB
- Determine the challenges in developing gene therapy clinical trials for inherited skin disorders

The first part of my thesis presents my work on the GENEGRAFT clinical trial (led by Professor Alain Hovnanian at INSERM in Paris) which is a phase I clinical trial awarded an FP7 grant in 2009. My role was to primarily characterise a UK patient cohort and preselect the most suitable patients for the trial. The trial sponsor was INSERM but KCL/GSTT was a clinical site for patient recruitment. At the time of commencing my research project there had only been one successful clinical trial of gene therapy in an inherited skin disorder (Mavilio et al, 2006). The details of this landmark study are detailed in Chapter 1, but one of the critical reasons for success was determined to be suitable patient selection. Patient selection for the GENEGRAFT study involved assessing the full UK cohort of patients at GSTT with RDEB and assessing according to molecular data and genotyping, immunofluorescence and western blot data analysis of amount of C7 expression, clinical features and in particular the presence of chronic wounds as well as the likely immune response to a *COL7A1* genetically corrected skin graft.

The second part of my research project involved working with Professor Adrian Thrasher and Professor Waseem Qasim at the Institute of Child Health in order to :

- Provide pre-clinical supportive data for a trial of intradermal allogeneic fibroblasts into unblistered skin with adults with RDEB

- Develop trial documents and work for application to ethics and MHRA for the trial

- Support clinical trial work and recruitment for other inherited skin disorders (Netherton syndrome)

Our own groups have focused on cell therapy, mainly fibroblast therapy. The rationale is based on studies showing intradermal injections of normal fibroblasts in mice can generate new C7 and AFs at the dermal epidermal junction (Wong et al, 2007). The main limitations of this approach are that these foreign cells are rejected within a few weeks only and a more sustained engraftment would be preferred. A complementary approach would be the use of gene therapy to correct autologous fibroblasts which could then be administered locally intradermally. The team at ICH already had a SIN lentiviral vector construct that had been approved for use in the UK to treat other conditions such as Wiskott-Aldrich syndrome. Although viral vector titre was relatively low, the data showed a reasonable transduction efficiency (up to 20%) and yet led to increased levels of C7 in RDEB fibroblasts. In addition I led the initial development of the clinical trial protocol as well as the preparation of trial documents facilitating a speedy application to the regulatory bodies.

Chapter 1

General Introduction and Aims of thesis

1.1 Skin structure

The skin constitutes the largest organ of the body and functions as a defensive barrier as well as acting as a homeostatic regulator of fluid, electrolytes and temperature. Full skin thickness varies between 0.5-4mm depending on the body area and consists of three distinct layers: the epidermis, dermis and subcutaneous fat.

The epidermis is an ectodermally derived stratified squamous keratinising epithelium and the principal cells within the epidermis are keratinocytes. The epidermis is highly specialised epithelium that is subdivided into 4 layers including the innermost basal cell layer, spinous layer, granular layer and outermost stratum corneum. Basal keratinocytes committed to terminal differentiation migrate upwards and initiate the process of keratinisation. The epidermis is consistently renewed every 3-4 weeks due to an ever-present supply of epidermal stem cells located within niche areas in the epidermis.

The underlying dense connective tissue dermal layer account for majority of skin's tensile strength and is composed of hair follicles, sweat glands, sebaceous glands and vascular networks embedded in a dense extracellular matrix (ECM). Collagens are the main structural component and provide a supporting scaffold for extracellular matrices of connective tissues. They are typically composed of a collagen triple helical domain which may be further sub-divided according to their structural and functional characteristics (Shaw and Olsen, 1991). The main cell type in the dermis is fibroblasts, important in the synthesis and secretion of the ECM.

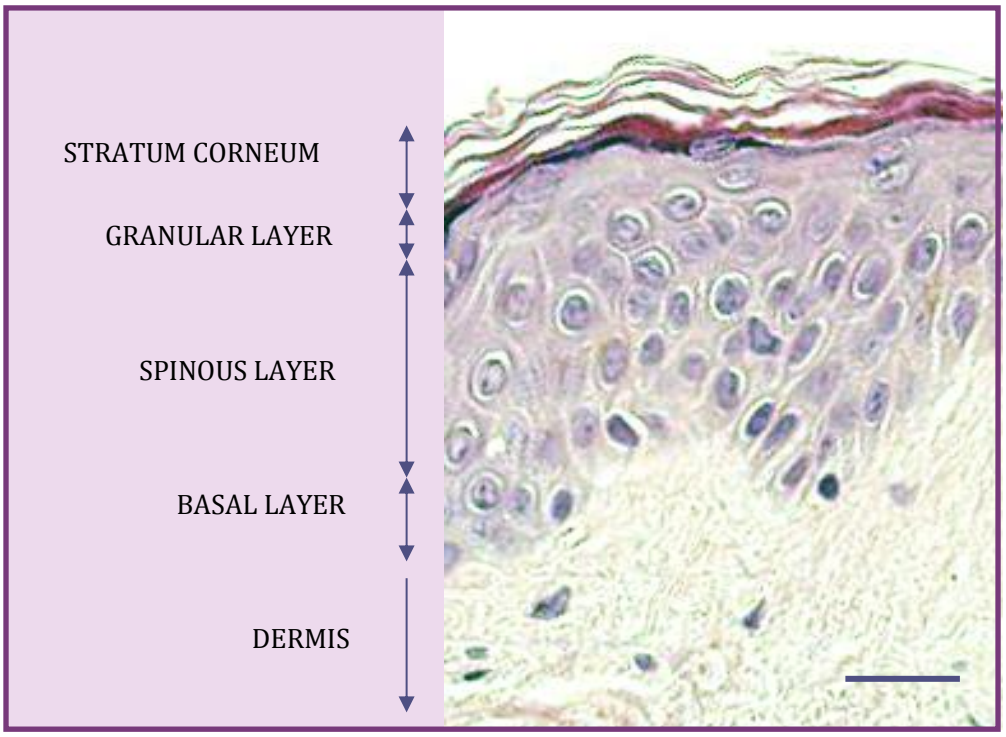


Figure 1.1 Histological appearance of normal skin

Normal human skin using hematoxylin and eosin staining, bar = 50 μm. The epidermis is composed of four layers as shown (the outermost cornified layer, the stratum corneum, the granular layer, spinous layer and the innermost proliferating basal layer).

1.2 Cutaneous basement membrane zone

The dermal-epidermal junction (DEJ) separates the epidermal and dermal layers. It is characterised by downward folds of epidermis called rete ridges which interdigitate with upward projections of the dermis called the dermal papillae. The DEJ consists of an extremely complex network of interconnecting intracellular and extracellular proteins, termed the cutaneous basement membrane zone, that has a typical morphological appearance at the ultrastructural level (Eady and Dunnill, 1994). Within this zone different macromolecules including hemidesmosomes, anchoring filaments and anchoring fibrils all function to preserve skin integrity.

The basement membrane zone (BMZ) is only visualised via electron microscopy and consists of an electron dense zone named the lamina densa, and superiorly an electron lucent zone named the lamina lucida, above which lie the basal keratinocytes and keratin intermediate filaments. Keratin 5 and 14 are the predominantly expressed keratins within basal keratinocytes (Lane et al., 1992).

The keratin intermediate filaments interact with two of the intracellular proteins found in the BMZ are Bullous Pemphigoid Antigen -1, BPAG1e (also known as BP230) and plectin. BP230 is an intracellular protein consisting of an N terminal head domain (plakin domain) and a C terminus possibly involved in binding keratin filaments (Tang et al., 1996). More critical for hemidesmosome assembly is plectin. Plectin is a large 200-nm-long protein found in the innermost portion of hemidesmosomes. This directly links intermediate filaments (IF) to both β4 integrin and to the 180-kDa bullous pemphigoid antigen (Koster et al., 2003, Nievers et al., 2000). At its C-terminus, plectin anchors IF. The N terminus harbours a highly conserved actin binding domain (ABD), followed by nine spectrin repeats (Sonnenberg and Liem, 2007) and binds to actin, β4 integrin and type XVII collagen (also known as Bullous Pemphigoid Antigen-2), BPAG2 or BP180. Type XVII collagen is a transmembrane protein which is mostly composed of a large extracellular C-terminus domain that is made up of 15 collagenous domains. Another important component of the hemidesmosomal outer plaque is α6β4 integrin. The β4 subunit has an intracytoplasmic tail domain which interacts with the hemidesmosmal proteins BP230, plectin and the cytoplasmic domain of type XVII collagen. The 120 kDa extracellular domain forms part of the anchoring filaments and binds to the α6 integrin subunit and laminin-332 and crosses the lamina lucida to anchor into the lamina densa. Collectively these proteins constitute the hemidesmosome-anchoring filament adhesion complex. The integrins and BP180 also connect to laminin 332, previously known as laminin 5, and along with laminin-311 contribute to forming anchoring filaments (Uitto and Pulkkinen, 1996).

Laminin-332 is a heterotrimer of 3 polypeptide subunits, α3, β3 and γ2, each encoded by the *LAMA3*, *LAMB3* and *LAMC2* genes, respectively. Being a major component of anchoring filaments, laminin-332 is located primarily in the lower lamina lucida and lamina densa (Masunaga et al., 1996). The connection between these proteins and other ubiquitous proteins in the BMZ such as nidogen, type IV collagen and laminin-511 form the underlying lamina densa (Yurchenco et al., 2002). From the lamina densa, large band like structure loop down into the interstial dermal matrix and back up to the lamina densa. These structures are called anchoring fibrils and their main constituent is type VII collagen (C7) (Shimizu et al., 1997, Burgeson et al., 1990).

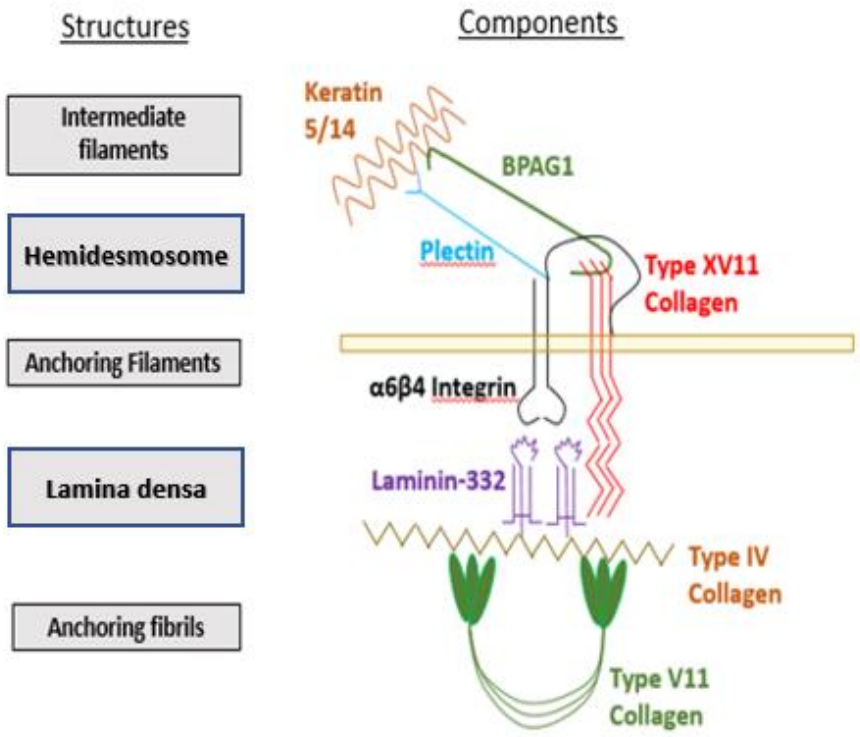


Figure 1-2 Schematic representation of the structures and components of the basement membrane zone.

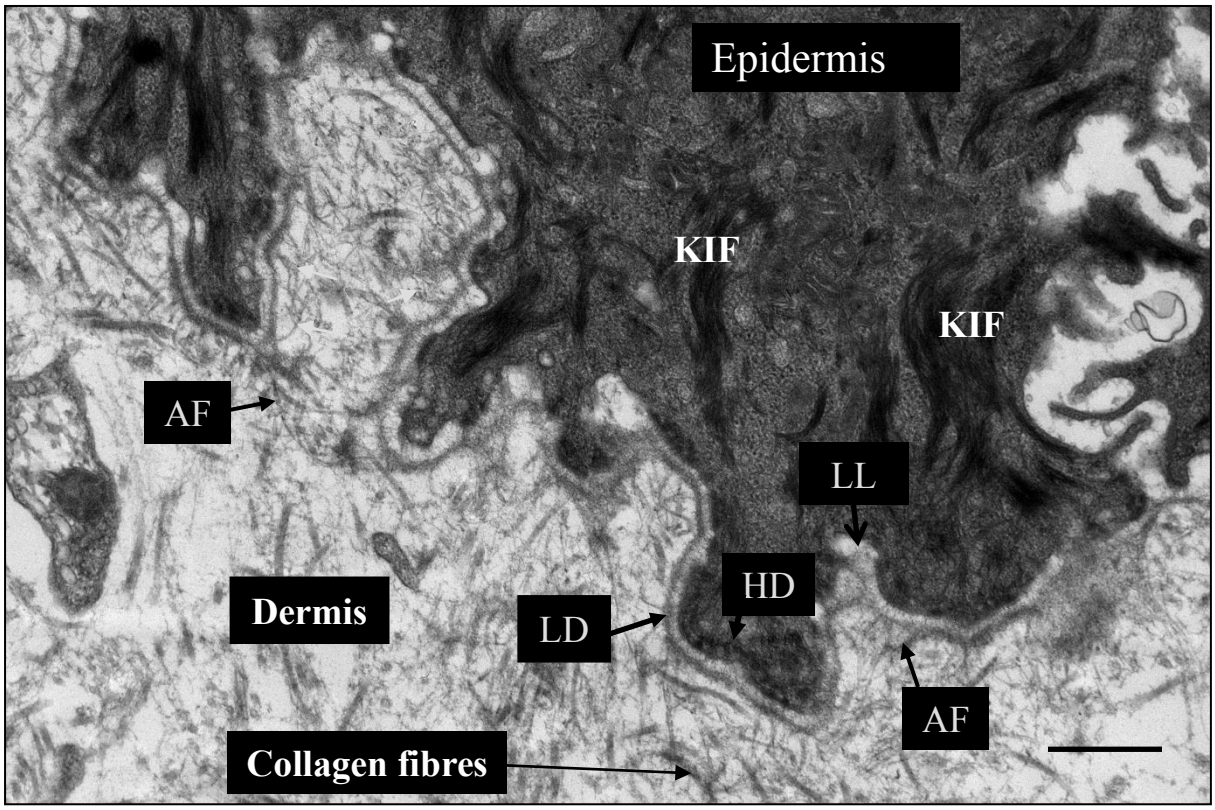


Figure 1-3 Transmission Electron Microscopy of the basement membrane zone in normal skin

HD = hemidesmosome, LD = lamina densa, LL = lamina lucida, AF = anchoring fibril, BMZ= basement membrane zone, KIF = keratin intermediate filament. Scale bar = 0.5 µm

1.3 Type VII Collagen

Type VII collagen (C7) is a genetically distinct collagen found predominantly in the basement membrane zone of the stratified squamous epithelia of the skin, mucous membranes and cornea (Wetzels et al., 1991). C7 is mainly synthesised and secreted by epidermal keratinocytes and also to a lesser degree by dermal fibroblasts (Bruckner-Tuderman et al., 1987).

C7 is known to be the main component of anchoring fibrils, structures anchoring the basement membrane zone to the underlying papillary dermis (Sakai et al., 1982). C7 is composed of three identical α chains known as pro-collagen. Each chain is composed of a central triple helical domain (triple helix). This large helical collagenous domain is composed of Gly-X-Y repeats.

In contrast to other interstitial collagens I-III, C7 has a number of deletions and insertions as well as a 39-amino acid non-collagenous insertion in the middle of the collagenous domain postulated to confer flexibility to the molecule allowing anchoring the lamina densa to underlying dermis (Christiano et al., 1994a, Järvikallio et al., 1997). Once secreted these monomers aggregate to form homotrimeric triple helical molecules and are then assembled in anti-parallel dimers with overlapping carboxy-terminal ends (Chen et al., 2001). During this process, the NC2 domain is proteolytically cleaved off. Subsequently the dimers are stabilised by interchain disulphide bonds and then assemble laterally to form anchoring fibrils, providing a stable attachment between the lamina densa and papillary dermis.

C7 is composed of a larger (~145kDa) amino terminal non-collagenous domain (NC-1), a central triple helical domain and the smaller (~30kDa) carboxyl terminal non-collagenous domain (NC-2). The NC1 domain contains multiple sub-units with homology to known adhesive molecules including fibronectin type III-like repeats and the A domain of von Willebrand factor (Chen et al., 1997). This larger NC1 domain is known to react with other extracellular matrix components including fibronectin, laminin-332, type I collagen (Villone et al., 2008), and type IV collagen (Burgeson et al., 1990).

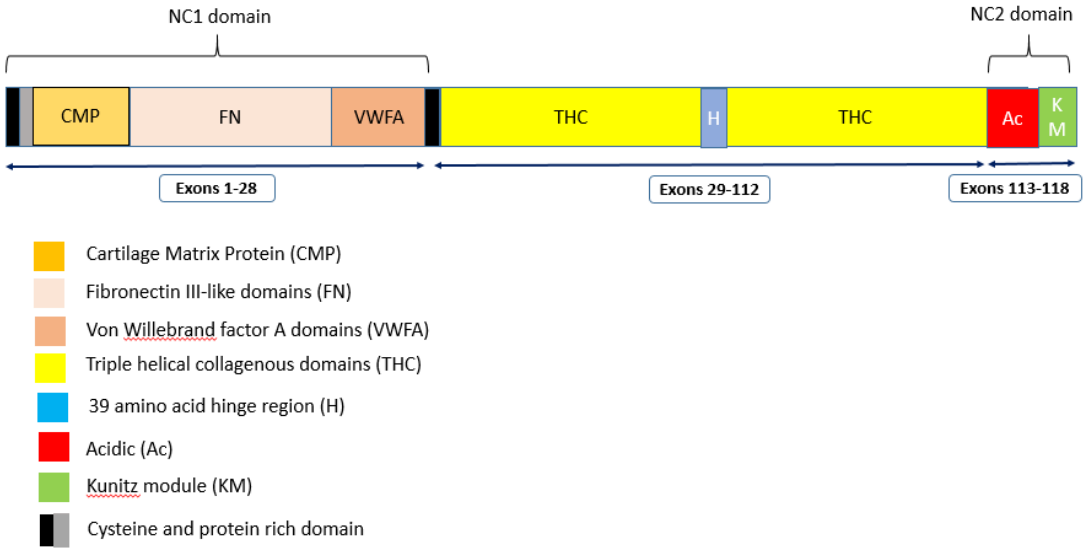


Figure 1-4 The domain structure of C7 and the encoding COL7A1 exons

The *COL7A1* gene is located on chromosome 3p21. The genomic structure was first fully described in 1994 and was demonstrated to have 118 exons, more than any other previously described gene (Christiano et al., 1994b). The resultant messenger RNA (mRNA) transcript is ~8.9 kb in size and is translated to give a precursor polypeptide, procollagen type VII (α1), ~300kDa composed of 2944 amino acids (Christiano et al., 1994a). The full length cDNA for type VII collagen contains 8833 nucleotides encoding 2944 amino acids. Mutations in *COL7A1* underlie all types of dystrophic epidermolysis bullosa (Uitto et al., 1994). There is also an acquired form of this disease that can result from an autoimmune response to C7 (Woodley et al., 1984).

1.4 Epidermolysis Bullosa and related skin fragility disorders

1.4.1 The classification of Epidermolysis Bullosa

Epidermolysis bullosa is a group of blistering disorders resulting from mutations in one of 18 genes encoding extracellular proteins found in the cutaneous basement membrane zone (BMZ). Mutations in one of these genes leads to absent or reduced protein levels or the production of aberrant non-functioning proteins, and therefore disruption to the integrity of the DEJ. EB was first formally classified according to electron microscopic features in 1962 (Pearson, 1962) and since then has been further characterised according to clinical phenotyping, immunofluorescence mapping and molecular diagnostics. According to the 2008 International consensus meeting on epidermolysis bullosa (EB), epidermolysis bullosa can be classified into 4 major subtypes: epidermolysis bullosa simplex (EBS), junctional epidermolysis bullosa (JEB), dystrophic epidermolysis bullosa (DEB) and Kindler syndrome (Fine et al., 2008). The subtypes can mostly be distinguished by the level of cleavage and blister formation. In EBS, the level of separation occurs at the suprabasal layer, in JEB at the lamina lucida and in DEB at the deepest level, the lamina densa within the upper papillary dermis. Kindler syndrome is

unique with blistering arising in multiple levels within the BMZ rather than within a discrete plane. This rare autosomal recessive blistering disorder with only 250 cases reported arises from premature termination of translation of kindlin-1 protein caused by mutations in the *FERMT1* gene (Jobard et al., 2003). Expression of kindlin-1 is localised to basal epidermal keratinocytes where it functions as a focal adhesion adaptor protein. The latest classification published in 2014 progressively divides each subtype according to phenotype, mode of transmission, ultrastructural site of cleavage and associated findings, target proteins, mutational type and finally by specific mutation present (Fine et al., 2014); this has been referred to as the "onion skin" diagnostic algorithm. A simplified version useful in clinical practice is summarised in Table 1.1.

EB type	**EB Simplex**		**Junctional EB**		**Dystrophic EB**		**Kindler syndrome**
Major subtype	Suprabasal	Basal	Generalised	Localised	Dominant	Recessive	Generalised
Level of cleavage	Intraepidermal		Lamina lucida		Sublamina densa		Mixed
Target protein(s)	Transglutaminase 5; plakophilin 1; desmoplakin; plakoglobin	Keratins 5 and 14; plectin; exophilin 5 (Slac2-b); bullous pemphigoid antigen 1	Laminin-332, collagen XVII; α6β4 integrin; α3 integrin subunit	Laminin-332, Type XVII collagen, α6β4 integrin	Type VII collagen		Fermitin family homologue 1 (kindlin)
Mutated genes	*TGM5, DSP, JUP, PKP1*	*K5, K14, PLEC, EXPH5, DST*	*LAMA3, LAMB3,LAMC2*	*LAMA3, COLXVII, ITGB4*	*COL7A1*		*FERMT1*
Mode of transmission	AD, AR		AR		AD	AR	AR
Skin distribution (predominant)	Generalised (some sparing of palms and soles)	Palms and soles	Generalised	Localised	Generalised	Generalised	Generalised
Clinical features	Blisters, erosions, crusts, focal/diffuse keratoderma usually minimal extracutaneous involvement, growth retardation		Blisters, crusts, GU involvement, caries, ocular, respiratory tract involvement	Blisters, milia, dystrophic nails, dental caries,	Blisters, milia, atrophic scarring, pseudosyndactyly, soft tissue oral involvement, oesophageal strictures		Poikiloderma, photosensitivity, skin fragility, caries, pseudosyndactyly

Risk of SCC	None	18-25% lifetime risk	Rare	Rare	++ Main cause of death	Increased risk after aged 30 years
Death due to EB	None; Uncommon in EBS Generalised	Death within first 2 years (JEB-severe generalised)	Rare	Rare	Usual life expectancy early 30s RDEB severe generalised (metastatic SCC)	Uncommon

Table 1.1 Different subtypes and clinicopathological and molecular subdivision of EB

Table showing the summary of clinical findings, associated morbidity and mortality, target gene, mutated protein and level of blister cleavage according to EB subtype (adapted from Fine *et al* 2014). Abbreviations: AD = autosomal dominant, AR = autosomal recessive, GU = Genitourinary, EBS = Epidermolysis bullosa simplex, JEB = Junctional epidermolysis bullosa, RDEB = Recessive dystrophic epidermolysis bullosa, SCC = squamous cell carcinoma

1.4.2 Incidence of inherited epidermolysis bullosa

In the United States, the incidence of all subtypes of EB is estimated at 20 per million live births, with a prevalence of 8 per 1 million of the population. Specifically RDEB is thought to affect one in every 1 in every 100,000 newborns in the United States (Pfendner et al., 2001). A study in Scotland estimated point prevalence to be approximately 32 per million of EBS, 26 per million of DEB and 0.4 per million in JEB (Horn et al., 1997). In all, there are approximately 500,000 people globally who have EB.

1.4.3 Epidermolysis bullosa simplex

Epidermolysis bullosa simplex (EBS) is a clinically heterogeneous group of disorders characterized by intraepidermal blistering resulting from mutations in genes encoding for keratinocyte intermediate filaments; most commonly keratin 5 (K5) and keratin 14 (K14) (Fine et al., 2014). The most common subtypes are localised, generalised intermediate and generalised severe. The phenotype is predominantly mild although Generalised Severe EBS (GS-EBS) is frequently associated with marked morbidity in infancy and childhood and, in rare cases, may result in death in early childhood (Sathishkumar et al., 2016). Recently further work has delineated rare subtypes of EBS and demonstrated the role of other important genes and target proteins, such as *EXPH5* encoding exophilin-5 (Liu et al., 2014, Pigors et al., 2014, McGrath et al., 2012) and *DST* encoding BP230 (Groves et al., 2010). Mutations in *PLEC* and *TGM5* as well as the desmosomal genes *PKP1*, *DSP* and *JUP* may also underlie a spectrum of EBS phenotypes (Indelman et al., 2005, Bolling et al., 2010a, Bolling et al., 2010b, Pigors et al., 2011).

1.4.4 Junctional epidermolysis bullosa

Junctional epidermolysis bullosa (JEB) results from mutations in genes encoding for proteins forming critical components of the hemidesmosome and anchoring filaments and leads to intra-lamina lucida blistering. JEB can be a devastating disorder in the generalised severe form and affected individuals rarely survive beyond the age of two years. Homozygous or compound heterozygous mutations occur in genes encoding for laminin 332 (*LAMA3, LAMB3, LAMC2*), integrin α6β4 (*ITGA6, ITGB4*) and type XVII collagen (*COL17A1*). The majority of the mutations occur in *LAMB3* but specific phenotypes are associated with different gene pathology, such as JEB with pyloric atresia in which mutations are found in *ITGB4* or, less frequently *ITGA6*.

1.4.5 Kindler syndrome

Kindler syndrome is a recently described additional EB subtype and is due to premature termination of translation of fermitin family homologue 1 (also known as kindlin-1 protein) caused by mutations in *FERMT1* (also known as *KIND1*) (Ashton et al., 2004, Jobard et al., 2003). This is an autosomal recessive disorder characterised by skin fragility, photosensitivity, poikiloderma, skin atrophy and scaling.

1.4.6 The molecular basis of DEB

Dystrophic epidermolysis bullosa (DEB) is characterised by a sub-lamina densa plane of blistering resulting from abnormal C7 synthesis and aberrant anchoring fibrils(Bruckner-Tuderman et al., 1989). Electron microscopy shows that AFs are absent, reduced or abnormal in structure or morphology (Hanna et al., 1983, Bruckner-Tuderman et al., 1990). Disruption of anchoring fibrils due to mutations in C7 is a characteristic of dystrophic EB.

DEB is therefore due to mutations in the gene encoding for type VII collagen, *COL7A1*. The first mutations in *COL7A1* were first reported in 1993 (Christiano 1993). Currently the International Dystrophic Epidermolysis Bullosa patient registry contains detailed information on 669 DEB patients, of which 71 cases are unpublished, and their 425 *COL7A1* mutations (van den Akker et al., 2011). Globally, however, the total number of *COL7A1* mutations identified by research and diagnostic laboratories probably exceeds 2000. The autosomal dominant form of the disease, dominant dystrophic epidermolysis bullosa (DDEB) is due to missense mutations in the Gl-X-Y repeat sequence (Dang and Murrell, 2008, Uitto et al., 1994). The autosomal recessive form, recessive dystrophic epidermolysis bullosa (RDEB), is generally caused by bi-allelic mutations that result in a premature termination codon (PTC) (i.e., nonsense mutations, frame-shifting deletions, insertions/duplications, indels, and certain splice-site mutations). As a result of mRNA degradation through nonsense mediated decay, these patients would have scanty or absent levels of C7 resulting in a severe clinical phenotype. Compound heterozygosity including only one non-PTC mutation would usually result in the production of varying quantities of C7 which may be partially functioning. Typically, this combination of mutations results in a milder or intermediate clinical phenotype.

1.4.7 Recessive dystrophic epidermolysis bullosa

Patients with generalised forms of RDEB (sev) present with severe cutaneous involvement. The condition is characterised by severe trauma-induced blistering and skin erosions from birth or early infancy. Wound healing is often slow, leading to chronic erosions, secondary infection and progressing to widespread, mutilating scars and contractures. Milia are often present. Trauma-prone areas such as hands, feet,

knees, elbows and bony prominences are most commonly affected. Recurrent wound infections are a common problem within extensive areas of eroded skin (Pillay, 2008).

RDEB affects mucous membranes and as such, gastrointestinal complications can range from microstomia, oral mucosal fragility, oesophageal strictures to blistering anal margins. Constipation frequently occurs. Oesophageal erosions, web and strictures cause severe dysphagia which further exacerbates the malnutrition (Azizkhan et al., 2006) and patients often require multiple oesophageal dilatations under fluoroscopic guidance. Malnutrition is therefore common and compounded by the additional nutritional requirements as a result of the continuous disruption skin integrity. Malnutrition is further exacerbated by chronic anaemia, often with an iron deficiency profile. Although the precise aetiology has not been fully explained, it has been postulated that the main causes of this iron deficiency anaemia is continuous blood loss from open wounds, poor nutritional intake and anaemia of chronic disease (Mellerio et al., 2007). Ophthalmic involvement is common, in particular corneal blister formation occurs. Symblepharon formation has a higher incidence in RDEB compared to other subtypes of EB and approximately half of all people with RDEB have some degree of ectropion (McDonnell and Spalton, 1988). Genitourinary tract involvement is less common and mostly reported in JEB. Urethral meatus stenosis is the commonest genito-urinary complication, occurring in 8% of RDEB patients (Rubin et al., 2007). Severe constipation in patients with RDEB may also result in urinary outflow obstruction with hydroureter and hydronephrosis.

1.4.8 RDEB, severe generalised (RDEB sev-gen)

This subtype was previously known as Hallopeau-Siemens recessive DEB (HS-RDEB) (Fine et al., 2008) and is one of the most severe subtypes of EB associated

with high morbidity and premature mortality. Electron microscopy shows absent AFs at the DEJ. The cutaneous and extracutaneous features are discussed above. The chronic wounds and widespread mutilating scar formation cause considerable suffering and have a severe impact on the quality of life. Chronic scarring of the digits results in fusion and contraction referred to as mitten hand deformity (McGrath et al., 1992). Mucous membranes are also affected. Oral involvement leads to ankylostomia and microstomia as a result of scarring and skin fragility. Oesophageal involvement leads to dysphagia and obstruction because of stricture formation. Malnutrition is secondary to these complications as well as the increased nutritional requirements as a result of skin barrier failure. Chronic constipation, painful defaecation and faecal impaction frequently contribute to malnutrition and failure to thrive.

Figure 1-5 Trunk and limb erosions and chronic wounds in an adult patient with RDEB severe generalised showing extensive ulceration across the torso (courtesy of Professor J A McGrath)

One of the main features of this subtype is the high incidence of early-onset squamous cell carcinoma (Mallipeddi, 2002). These tumours have a different pathophysiology to classical ultraviolet induced SCC and behave aggressively leading to early

mortality. One of the explanations for SCC development has been the persistent wounding and scarring suffered by those with RDEB, which can theoretically deplete basal stem cell progenitors and stimulate a pre-malignant microenvironment (Smoller et al., 1990). Additional factors affecting cell cycle regulation such as mutated p53, reduced expression of insulin growth factor-binding protein 3 and elevated matrix metalloproteinase (MMP-7) expression have been linked to SCC in RDEB patients (Martins et al., 2009). Data shows that the median survival from the diagnosis of the first SCC is only 5 years and nearly 80% will have died of metastatic SCC despite aggressive surgical resection (Fine et al., 2009, Pfendner and Lucky, 1993, Ayman et al., 2002)

1.4.9 RDEB, generalised other

This subtype was previously known as RDEB non-Hallopeau-Siemens. The clinical phenotype is milder and more limited than RDEB-sev-gen. Electron microscopy shows reduced or rudimentary AFs as opposed to absent. Extra-cutaneous features including glomerulonephritis, renal amyloidosis, IgA nephropathy, cardiomyopathy, delayed puberty and osteoporosis are absent. Although the risk of developing skin cancer is also comparatively less, aggressive SCCs still represent a significant cause of morbidity and mortality (Fine et al., 2008).

1.4.10 RDEB, inversa

The inversa type of RDEB is rare and was first described by Gedde-Dahl Jnr in 1971 and is characterised by generalised blistering from birth and during early infancy (Gedde-Dahl, 1971). Later in childhood, the affected areas concentrate to the flexural sites as opposed to the exposed friction-prone sites. Common sites include the groins,

axillae, neck and lumbar area (Fine et al., 2008). Mucosal sites particularly the cornea and oesophagus are frequently severely affected.

Figure 1-6 Ulceration and contractures of the fingers in patients with severe RDEB

(A) chronic scarring, dyspigmentation and mitten hand deformity in adult patient with severe RDEB and (B) severe ulceration of the hand with a degloved finger (courtesy of Prof Jemima Mellerio)

1.5 Management of RDEB

At present only palliative therapies are available for patients with RDEB. Appropriate wound care and daily dressing changes are the mainstay of management. A multidisciplinary approach to manage pain control, dental hygiene, chiropody, ocular complications, joint contractures as well as psychosocial support and care is absolutely essential. The Dystrophic Epidermolysis Bullosa Research Association (DebRA) has listed a number of recommendations including the use of non-adhesive dressings only, cautious hand manipulation and exercise to prevent contractures. In addition to wide varieties of non-adhesive dressings available, tissue engineered skin substitutes are also commercially available. Recently human amniotic membrane grafts have also been trialled (Lo et al., 2010) utilising their biological properties to promote wound healing. In a retrospective, proof-of-concept study, amniotic membrane grafting was efficacious in promoting the healing of non-healing wounds in EB with a reduction in pain but complete re-epithelialisation was not achieved. Before the genetic basis of dystrophic EB was discovered, ultrastructural studies indicated possible collagen degradation and phagocytosis of collagen fibrils in areas of blistering in RDEB skin. Early attempts at systemic treatment for RDEB focused on inhibiting collagenase. Anecdotal reports of systemic therapies have cited treatment with tumour necrosis factor- α (TNF-α) inhibitor etanercept (Gubinelli et al., 2010), vitamin E (Ayres 1986), phenytoin (Rogers et al., 1983, Wirth et al., 1983), mycophenolate mofetil (Eldarouti et al., 2013), oral trimethoprim (Lara-Corrales et al., 2012) and recently systemic granulocyte colony-stimulating factor (G-CSF) (Fine et al., 2015); but none has shown significant clinical impact. Pre-clinical studies have been reported aiming for cell, drug, protein or gene therapies in order to increase levels of C7 at the DEJ and ameliorate the phenotype.

1.5.1 Cell therapies

Cellular therapies to repair and restore a defective epithelium are an attractive option in the management of RDEB. Initial studies were based on the treatment of burns patients and the development of composite skin grafts, including a dermal component such as de-epidermized cadaveric dermis, biopolymers, or artificial scaffolds allowed for further refinement of cultured skin for clinical use (Auger et al., 2004, Ojeh et al., 2001). Cultured epidermal keratinocyte grafts were trialled in EB in the early 1990's but success was limited due to the high associated risks of graft infections (McGrath et al., 1993, Hill et al., 1992). In one study, cultured epidermal autograft (CEA) was manufactured by taking a full-thickness biopsy specimen of skin from an RDEB subject and culturing keratinocytes to confluence. The CEA was then grafted onto a selected area of ulceration with epithelialisation observed 2 weeks later (Shinkuma et al, 2014). Intradermal administration of cellular products is less invasive and fibroblasts able to secrete ECM are a possible option. Intradermal allogeneic fibroblasts have been used as a therapy for RDEB in both phase I and phase II clinical trials in human subjects (Wong et al., 2008, Petrof et al., 2013, Venugopal et al., 2013). The initial trial by Wong *et al* (2008) assessed the effect of single intradermal injections of neonatal-derived allogeneic fibroblasts (5×10^6 cells injected into the superficial dermis over ~ 1 cm2) to intact skin on the backs of 5 patients with RDEB. Clinically 3 subjects had RDEB-(sev-gen) with almost completely undetectable levels of C7, whereas 2 subjects had RDEB-(gen-intermediate) with reduced levels of C7 protein still detectable. All 5 subjects demonstrated a 1.5-2 fold increase of C7 at the DEJ at 2 weeks and 3 months after injection. Concomitant cultured autologous fibroblasts and parental fibroblasts were administered as controls, but it was the allogeneic cells that induced greatest expression of C7. Subsequent studies showed

that this induction of C7 at the DEJ with subsequent improvement of dermal-epidermal blistering at the site of injection was achieved and remained stable for 3 months in most cases and up to 9 months in one subject with RDEB with no clinic-pathological inflammatory response observed (Nagy et al., 2011). Gene expression profiling showed that allogeneic fibroblasts injected directly into the dermis increased expression of, among other factors, heparin binding-EGF-like growth factor (HB-EGF). HB-EGF has been shown to increase *COL7A1* gene expression in normal or RDEB keratinocytes and fibroblasts, which in turn leads to an increase in expression of rudimentary AFs, which stabilise the DEJ and ameliorates the blistering tendency. Limitations of this approach are the clearance of allogeneic cells by host immune response, as fluorescent *in situ* hybridizsation (FISH) for the detection of donor Y-positive cells in a female recipient demonstrated clearance of allogeneic fibroblasts 2 weeks following administration. Furthermore, the therapy was only effective in patients with some level of expression at the DEJ. It was proposed that either an initial burst of C7 from the introduced fibroblasts or a paracrine effect exerted on the patients' keratinocytes stimulating production of mutant protein may have contributed to the partial improvement seen. Stimulation of endogenous C7 expression by patients' own cells has been associated with a heparin EGF-like growth factor (HB-EGF) induced by fibroblast injections, which in turn leads to an increase in expression of the AF forming protein predominantly by the keratinocytes. These findings were further supported by a phase II double-blind randomised controlled trial of allogeneic fibroblasts administered to chronic wounds in RDEB (Petrof et al., 2013). Petrof et al (2013) demonstrated that a single administration of 5×10^6 allogeneic fibroblasts could increase the rate of erosion healing in RDEB up to 28 days but not thereafter. A further phase II study showed equal increase in wound healing and *COL7A1* expression

following intradermal administration of allogeneic fibroblasts and a 2% albumin suspension solution (Venugopal et al., 2013). The benefits of administering fibroblasts as a cell therapy include the ease of access to commercially available fibroblast products, the robust nature of fibroblasts in cell culture and an excellent safety profile when used for other clinical indications.

Cell therapies for RDEB have now expanded to the use of bone marrow derived cells, including mesenchymal stromal cells (MSCs) and haematopoietic stem cells (HSCs). It is now known that bone marrow (BM) cells contribute a proportion of cells in the skin that have roles in skin development, homeostasis, repair and regeneration. BM cells that are present in skin can be derived from either haematopoietic or mesenchymal stromal cell (MSC) populations. Some sub-populations of the BM cells can differentiate into skin epithelial cells (keratinocytes) (Fathke et al., 2004a, Badiavas et al., 2003, Borue et al., 2004) and also provide fibroblast-like cells to the dermis, although their precise nature is not fully understood. Human and murine studies involving transplantation of sex-mismatched or genetically-tagged BM cells have shown that keratin-positive BM-derived cells can be found in skin epidermis, hair follicles and sebaceous glands, sites that contain skin stem cell niches (Brittan et al., 2005, Krause et al., 2001). With respect to EB, Chino *et al* (2008) were first able to demonstrate the capacity of bone marrow cells to contribute dermal fibroblasts and prolong survival following the transfer of marrow cells from 8-week-old green fluorescent protein-positive (GFP+) mice into the circulation of 13-day-old *col7a1*-/- embryos (E-BMT)(Chino et al., 2008). Subsequently subsets of BM-derived cells were transplanted into neonatal *col7a1*-/-mice, with the result that bone marrow-derived cells migrated to the skin, mediated C7 deposition leading to prolonged survival of *col7a1* mice (Tolar et al., 2009). These observations of increased C7 in

skin BMZ following bone marrow transplant (BMT) thereby provided a rationale for BMT to be considered as a potential therapy for humans with RDEB.

The first bone marrow transplant in human subjects with RDEB were reported in 2010 (Wagner et al., 2010). Seven patients with RDEB were selected and 6 proceeded with allogeneic stem cell transplants following immunomyeloablative chemotherapy conditioning using busulphan, cyclophosphamide and fludarabine. Immunoprophylaxis against graft versus host disease using ciclosporin and mycofenalate mofetil was initiated and continued up to 100 days. In the majority of subjects (5 of 6 evaluable individuals), light microscopy demonstrated an increase in C7 at the DEJ. Clinically, there was a significant improvement with reduction in the amount of blisters and erosions on the skin and mucous membranes, and the physical benefits continued for at least 500 days after the BMT. Indeed, follow up for >6 years has shown sustained clinical benefits (Tolar et al, 2015). The associated high risk of mortality means that other safer alternatives should be explored, such as intravenous mesenchymal stromal cell infusions or reduced intensity conditioning (RIC) regimens of BMT. Combination conditioning has been reduced from using busulfan, fludarabine, and cyclophosphamide to combination therapy with fludarabine and low doses of cyclophosphamide and radiation. The most recent update has reported a further 20 patients with RDEB-sev-gen between 0.4 and 20 years who underwent allogeneic HCT (Tolar et al, 2015). The hematopoietic graft was HLA-matched related BM (n=10), unrelated BM (n=7) or umbilical cord blood (UCB)(n=3). In addition, a single infusion of third-party HLA-mismatched MSC was administered. For the entire cohort of engrafted, surviving subjects with RDEB thus far (N=18 of 26), 14 individuals demonstrated partial to marked biochemical and clinical improvement in mucocutaneous disease as evidenced by >50% reduction in affected body surface area,

and/or C7 expression. Although the reduced intensity conditioning regimen (RIC) had a significantly lower risk of morbidity and mortality compared with myeloablative conditioning (MAC) (2 year survival 90% with RIC compared with 69% with MAC), risks of therapy still remain significant necessitating other separate or adjunctive therapies. The incidence of graft versus host disease in this cohort although not specified was reported to be unexpectedly low, which may be due to the adjuvant MSC infusion or a specific feature of EB patients.

1.5.2 Protein therapy

The rationale for protein replacement consists of delivering recombinant human C7 protein locally or systemically. In 2004, Woodley *et al* demonstrated unexpected efficacy when intradermally injected human C7 was reported to incorporate stably into the BMZ of wild-type mice, as well as into regenerated human RDEB equivalent skin transplanted onto immune-compromised mice (Woodley et al., 2004a). Remington *et al* (2009) were also able to show incorporation of intradermally injected C7 into the BMZ of RDEB mice with restoration of AF structure rescuing the clinical phenotype (Remington et al., 2009). Although these mice developed antibodies to the newly administered protein, this did not preclude the development of functional anchoring fibrils. Although these antibodies were found in the circulation they did not bind to the DEJ, therefore unlikely to lead to clinically deleterious consequences (Woodley et al., 2004a). Unlike other collagens, C7 is a soluble protein and would therefore be possible to administer intravenously. This would be a far preferable option in RDEB which has widespread systemic involvement. More recently Woodley *et al* have confirmed that intravenously administered C7 can home to wounds in a murine model where it formed AFs and led to improved wound healing (Woodley et al., 2013). Before considering phase I studies of intravenous therapy pre-clinical safety and

toxicology studies in larger animals may be necessary in order to determine appropriate dosing in humans and to investigate the overall risks of the procedure with respect to thrombus formation, production of anti-C7 antibodies and C7 deposition in vital organs. Momentum had been building for a phase I trial of recombinant C7 protein (rC7) therapy for RDEB following the commercial backing of Shire's recent acquisition of Lotus Tissue repair specialising in the production of rC7. These plans have been put on hold due to severe adverse effects and toxicity encountered during testing in larger animal models (no further information available at the moment, Sept 2016). Furthermore, there have been recent data published regarding the half-life of C7 that would impact the schedule for protein therapies. Kühl et al. (2016) attempted to determine the half-life of C7 in the skin, tongue, and oesophagus of genetically altered mice that express C7 constitutively, but with its expression abrogated by genetic manipulation. Their results revealed a half-life much shorter than previously anticipated, some 30 days (Kuhl et al., 2016), although it is unknown whether this observation will be similar in human skin, and also in patients with continuous wound healing and epidermal renewal. Clinical trials in human subjects are needed to answer these questions.

1.5.3 Drug therapies

Drug therapies such as antibiotics with anti-inflammatory activity have been trialled with respect to the promotion of wound healing and reduction of blister formation in patients with EBS (Fine and Eady, 1999, Retief et al., 1999, Weiner et al., 2004, Veien and Buus, 2000). A proof-of-concept study in RDEB showed improved wound healing following oral trimethoprim compared to placebo (Lara-Corrales et al., 2012) but further studies are required to prove potential benefit. Newer therapeutic approaches are aiming to explore and exploit the observation of elevated TGF-β in acutely injured

RDEB skin. Indeed, TGF-β pathways may modulate disease variability. Observations of a monozygotic twin pair with RDEB presenting markedly different phenotypic manifestations, while expressing similar amounts of collagen VII, highlights the role of TGF-β in modifying disease severity (Odorisio et al., 2014). Genome-wide expression analysis in twins' fibroblasts showed differential expression of genes associated with TGF-β pathway inhibition. In particular decorin, a skin matrix component with anti-fibrotic properties, was found to be more expressed in the less affected twin. Longterm use of losartan (diminishing levels of TGFβ and IL6) in RDEB mouse models has been shown to reduce scarring and fibrosis (Nystrom et al., 2015) and is currently being assessed in RDEB in human clinical trials. Although there have been some limited studies using standard strategies aiming to target inflammatory cytokines, further exciting developments have utilised gene silencing mechanisms. Short-interfering RNAs (siRNAs) are double-stranded RNA molecules able to target and down-regulate specific genes and can be used to knockdown target gene expression, applicable for dominant disorders such as EB simplex and DDEB (Atkinson et al., 2011). Drug therapies that induce read-through PTC mutations have been developed as potential generic treatments for inherited disorders with nonsense molecular pathology, thus some cases of RDEB may also potentially be suitable for this approach. Cogan *et al* (2014) have showed that aminoglycosides (G418, gentamicin, and paramomycin) are able to induce PTC read-through and restore a functional full-length C7 by increasing the stability of C7 mRNA in RDEB keratinocyte cell lines (Cogan et al., 2014). Aminoglycosides such as geneticin, gentamicin and paromomycin when transiently delivered in an expression vector can mediate “readthrough” of nonsense mutations and restore C7 expression. This group has further described the generation of RDEB nonsense mutations by site-directed

mutagenesis, the constructs then being transfected into human 293 epithelial cells. Without aminoglycosides, these cells produced no C7. By contrast, treatment of the cells with aminoglycosides induced C7 expression between 10 and 80% of the level of C7 expressed in cells transfected with an expression vector for normal C7. These data suggest that aminoglycosides may provide benefit for the 10-15% of RDEB patients with nonsense mutations that create premature stop codons synthesising minimal or absent C7 (Cogan et al., 2014). Antisense oligonucleotides have been developed for RDEB to restore the open reading frame of COL7A1 through selective removal of the mutated exon, or by restoring correct splicing (Turczynski et al., 2012).

In summary promising pre-clinical studies for strategies using protein replacement (Woodley et al., 2004a), cell therapy (Woodley et al., 2007, Wong et al., 2008) and drug therapies (Shiozuka et al., 2010) are underway. The most considerable progress in human subjects has been made in the area of cell therapies for RDEB, using dermal fibroblasts (Wong et al., 2008) and most recently bone marrow-derived cells. Allogeneic cell therapy is limited by host immune response and cell rejection. Gene correction offers great hope for the delivery of autologous cells that would have a sustained engraftment and therefore longer lasting clinical benefit.

1.6 Gene replacement strategies

In 1989, Rosenberg *et al*. performed the first human gene therapy trial using a retrovirus to introduce the gene coding for resistance to neomycin into human tumour-infiltrating lymphocytes before infusing them into five patients with advanced melanoma (Rosenberg et al., 1990). Thereafter the first therapeutic gene therapy clinical trial was for SCID (Severe Combined Immunodeficiency) caused by adenosine deaminase (ADA) deficiency (Blaese et al., 1995) and X-linked Severe

Combined Immunodeficiency (SCID-X1). Since these early trials there have been continuous improvements in the effectiveness and safety of these viral vector technologies to become a significant platform for both basic and clinical research. Therapeutic efficacy has now been reported for anti-cancer therapies as well as inherited diseases. Inherited diseases treated successfully in clinical trials have included chronic granulomatous disease (Kang et al., 2011), Leber's congenital amaurosis (Bainbridge et al., 2008, Maguire et al., 2008), adrenoleukodystrophy (Cartier et al., 2012), β-thalassaemia (Cavazzana-Calvo et al., 2010), metachromic leucodystrophy (Biffi et al., 2013), Wischott-Aldrich syndrome (Aiuti et al., 2013), cystic fibrosis (Alton et al., 2013) and haemophilia B (Nathwani et al., 2011). The spectrum of acquired disorders currently being targeted with gene therapies is vast including neurodegenerative disorders, Alzheimers disease (Tuszynski et al., 2015), cancers (Buscail et al., 2015) and HIV (Mitsuyasu et al., 2009). Overview of progress made in gene therapy clinical trials is available on a searchable database at: http://www.wiley.co.uk/genmed/clinical. As of June 2012, 1843 trials had been undertaken worldwide in 31 countries (Ginn et al., 2013). Currently there are 1172 clinical trials actively recruiting patients for gene therapy phase I/II studies (www.clinicaltrials.gov 12th September 2016). Despite the large number of clinical trials to date, only one gene therapy product (Glybera®) has been approved for clinical applications (Yla-Herttuala, 2012). Alipogene tiparvovec (Glybera) is a gene therapy product approved in Europe under the "exceptional circumstances" pathway as a treatment for lipoprotein lipase deficiency (LPLD), a rare autosomal recessive disorder resulting in chylomicronemia and an associated increased risk of acute and recurrent pancreatitis, with potentially lethal outcome. The product is an intramuscular

injection of a recombinant adeno-associated viral vector (AAV) containing the Ser(447)X variant of the human lipoprotein lipase (*LPL*) gene (LPLSer(447)X).

1.6.1 Viral vector mediated gene therapy

Viruses are efficient biological vehicles that have sophisticated machinery to enable access to cells to exploit their own cellular mechanisms for replication. The use of viruses in gene therapy is achieved by harnessing their ability to integrate into the genome but avoid the viral genes leading to replication and cell death. The two components of viral vector gene delivery systems are a) the vector construct and b) the packaging elements which form the structural proteins, envelope and enzymes necessary to generate infectious particles.

The number of viruses under development as gene therapy vectors is expanding but the main classes are oncoretroviruses, lentiviruses, adenoviruses, adeno-associated viruses and herpes simplex-1 viruses. Only oncoretroviruses and lentiviruses can integrate into host cellular chromatin and would therefore be suitable for tissue with high cellular turnover to achieve reproducible and stable gene expression. Furthermore, retroviral and lentiviral vectors have not been shown to induce a significant host immune response in comparison to adenoviral vectors (Worgall et al., 1997). Vectors derived from the *retroviridae* family are nowadays among the most widely used. The *retroviridae* family comprise a group of positive-sense single-stranded (ss) RNA diploid viruses, roughly spherical in shape which stably integrate a cDNA copy of their RNA genome into the host cell chromosomes by use of reverse transcription and integration. Up to 70% of gene therapy clinical trials thus far have utilised viral vectors. Almost 20% of these vectors are gamma retroviral vectors. Retrovirus' have a unique life cycle (RNA genome-DNA genome - RNA - Protein)

and are divided into 7 genres: simple (alpha, beta, gamma or epsilon) and complex (delta, lenti and spira). The most commonly used are gamma retroviral vectors as well as lentiviral vectors.

1.6.1.1 γ-retroviral vectors

Moloney murine leukemia virus (MLV) based γ-retroviral vectors have been commonly used to transfer genes into dividing cells and was the vector used in the first successful gene therapy clinical trial in X-linked SCID (Cavazzana-Calvo et al., 2000). Limitations of oncoretroviral systems include poor translocation across intact nuclear membranes and a tendency for transgene 'silencing' over time (Fenjves et al., 1996). In addition, conventional murine retroviral vectors have been shown capable of mediating insertional mutagenesis in clinical gene therapy studies (Hacein-Bey-Abina et al., 2003). Retroviral vectors favour integration within 5kB of transcriptional start sites (Wu et al., 2003). Integration close to cellular proto-oncogenes can lead to the trans-activation of those genes through the action of strong enhancer/promoter elements contained within the viral long terminal repeat (LTR)(Lazo et al., 1990). Genotoxicity is thought to derive from the long distance, cis-acting activity of the long terminal repeat (LTR) U3 enhancer combined to a target site selection mechanism that preferentially integrates close to transcriptional start sites and transcriptional regulatory elements (Wu et al., 2003, Bushman et al., 2005). A strategy to prevent this LTR activity is to delete the enhancer/promoter sequences from the U3 region, termed self-inactivating (SIN) vector design (Yu et al., 1986). Another issue in the use of both gamma retroviral and lentiviral vectors is the risk of transgene silencing via repressive DNA epigenetic effects including histone modification and DNA methylation (Bestor, 2000). A recent clinical trial for X-linked chronic granulomatous disease (X-CGD) is an illustration of this mechanism having a negative impact on clinical trial outcome.

In this study, two young adults treated for X-CGD using an infusion of autologous CD34+ cells gene corrected *ex vivo* using a γ-retroviral vector (Ott et al., 2006). This viral vector contained a spleen focus forming virus (SFFV) promoter enhancer element within the LTR to drive the therapeutic transgene (gp91phox cDNA). After the initial resolution of bacterial and fungal infections, both subjects showed silencing of transgene expression due to methylation of the viral promoter, and myelodysplasia with monosomy 7 as a result of insertional activation of ecotropic viral integration site 1 (EVI1). One subject died from overwhelming sepsis 27 months after gene therapy, whereas a second subject underwent an allogeneic HSC transplantation (Stein et al., 2010). These outcomes of trials using MLV based classical γ-retroviral vectors were a major stumbling block in the road towards the development of human gene therapy clinical trials. Over the last 5 years SIN lentiviral vectors have been developed and shown to be safer with reduced genotoxic potential.

1.6.1.2 Lentiviral vectors

The lentiviral vectors used for gene transfer are derived from the Human Immunodeficiency Virus (HIV). These are single stranded RNA viruses, capable of entry into mammalian cells and propagate via chromosomal integration (Barre-Sinoussi et al., 1983). The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilobases in length (Muesing et al., 1985). The HIV genome encodes at least 9 different proteins: (1) the major structural proteins gag, pol and rev; (2) the regulatory proteins tat and rev; and (3) the accessory proteins vif, vpu, vpr, and nef. A viral core is formed encapsulated by two distinct protein shells, and enveloped by host cell lipid membrane. There are seven stages within the life cycle of the virus: attachment, entry, reverse transcription, nuclear import, proviral integration, transcription, nuclear export and translation viral assembly and budding.

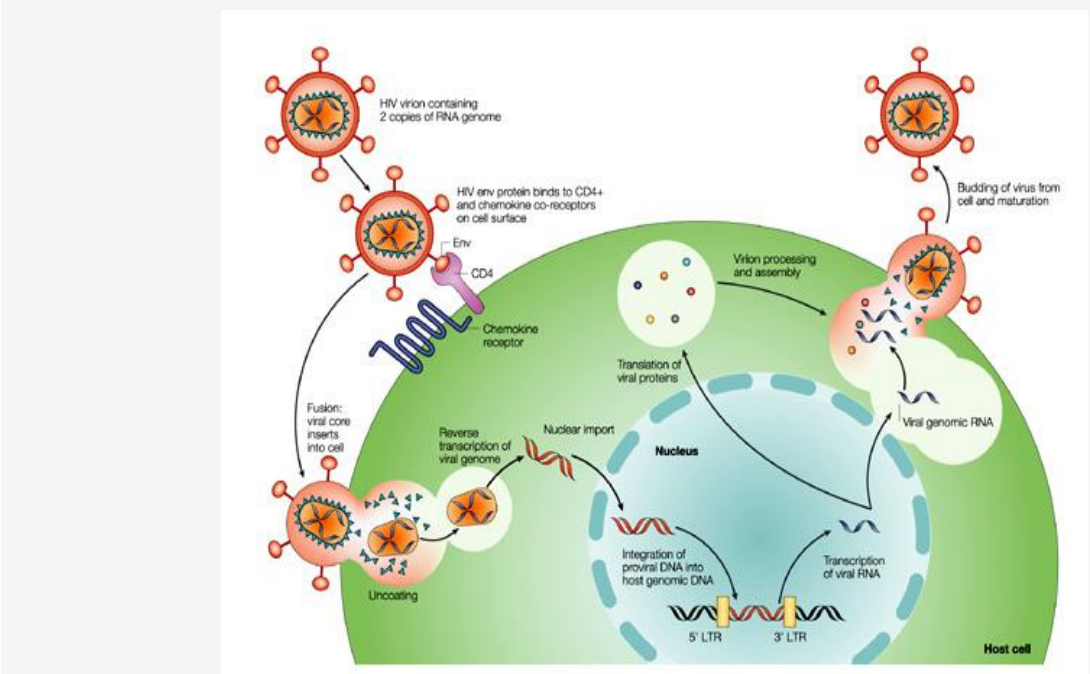


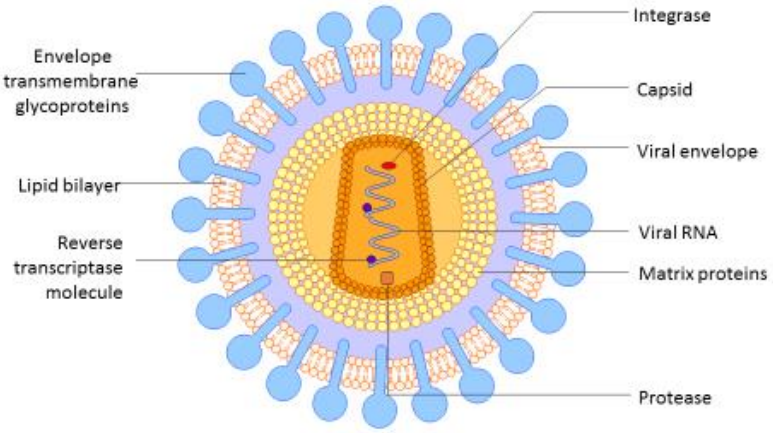
Figure 1-7 Illustration of the HIV-1 life cycle (adapted from Rambault 2004)

The viral proteins required for delivery into the nucleus and integration are carried into the cell by the virion, and appear to remain associated with the intracellular replication intermediates through the steps leading to integration.

Lentiviral vectors have been shown to be able to transduce quiescent or minimally stimulated cells (Zufferey et al., 1998a) in contrast to their γ-retroviral counterparts. This property relies on the use of a nuclear import pathway enabling the viral DNA to cross the nuclear membrane of the host cell, facilitated by the pre-integration complex (PIC) (Miller et al., 1997). This is important considering that for a longer term correction of phenotype, it is necessary to target the slow cycling stem cell populations. The main vectors used are derived from the HIV-1 virus and use a third generation HIV lentiviral vector system. In the second generation lentiviral vector system, five of the nine HIV-1 genes are eliminated, retaining genes for virion

structural proteins and enzymes (gag/pol) and genes relating to transcriptional and post-transcriptional functions (tat and rev). These vectors are produced with a 3 plasmid packaging system. The latest third generation vectors have further HIV-1 genes deleted leaving only gag, pol and rev and are produced using a four-plasmid set. This vector generation has been designed using a chimeric LTR (long terminal repeat) ensuring transcription in the absence of tat (Dull et al., 1998a). As a result, this vector system possesses a higher safety profile and is more suitable for clinical applications (Montini et al., 2006). The trial using an HIV based, lentiviral vector system reported the successful treatment of two children with X-linked adrenoleukodystrophy (X-ALD) using a vector encoding the deficient peroxisomal adenosine triphosphate–binding cassette transporter protein ABCD1 (Cartier et al., 2012). Despite the use of a lentiviral vector that contained an internal viral LTR to drive transgene expression, no clustering of vector insertions in oncogenes or growth-related genes was observed. This study paved the way for further trials for a wide variety of disorders including Wiskott-Aldrich syndrome (Aiuti et al., 2013), metachromic leukodystrophy (Biffi et al., 2013), β thalassaemia (Cavazzana-Calvo et al., 2010) and ADA-SCID (Carbonaro 2012).

A



B

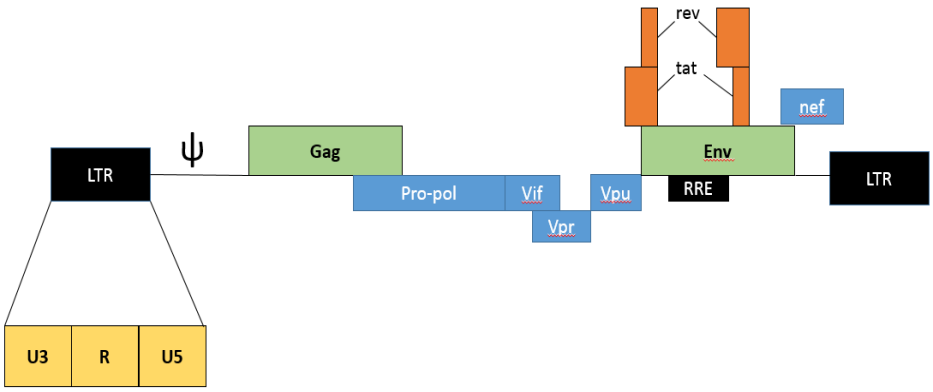


Figure 1-8 The HIV virion and genome organisation

(A) The structure of the HIV virion showing the viral envelope containing the matrix proteins and nucleocapsid. (B) The HIV genome organization is shown in this scheme from 5' to 3' end as found in the integrated provirus. The 5' long-terminal repeat (LTR) is subdivided in three functional regions; the U3 (HIV promoter), the R and U5 regions.The 5' LTR is followed by the packaging signal (ψ), which directs the specific packaging of the HIV genome into the lentivirus particles, and the Gag-Pro-Pol and Env genes. The distribution of the HIV-1 regulatory/virulence accessory genes *vif*, *vpr*, *vpu*, *nef*, *rev*, and *tat* is also shown. RRE represents the rev-response element.

The development of biosafety safeguards have led to the development of third generation and now fourth generation lentiviral vectors. The packaging unit of the first generation of HIV based vectors comprised all proteins except the envelope (Naldini et al., 1996a). The next generation of safety measures ensured deletion of four additional genes, encoding pathogenic virulence factors: *Vpr*, *Vif*, *Vpu* and *Nef*. (Zufferey et al., 1997). Third generation vectors conserve only three of the nine genes present in parental virus genome: *gag*, *pol* and *rev* (Dull et al., 1998b).

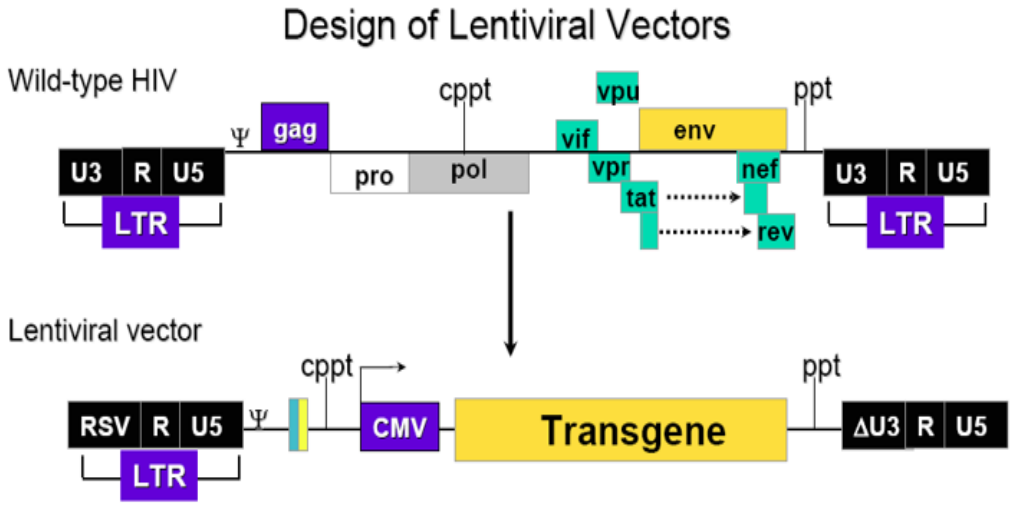


Figure 1-9 Schematic demonstrating the design of third generation lentiviral vectors.

Wild type vector showing major structural proteins gag, pol and rev; the regulatory proteins tat and rev; and the accessory proteins vif, vpu, vpr, and nef. The packaging signal (Ψ) and central polypurine tract (cppt) are indicated together with the U3, R, and U5 regions of the 3'- and 5'- long terminal repeats (LTR). The lentiviral vector shows the modified U3 region (ΔU3) in the 3'-LTR. Figure courtesy from Dr M. Antoniou.

Integration mapping has shown that HIV based lentiviral vectors integrate at gene rich sites but do not cluster around transcription start sites in the same manner as gamma retroviral vectors in haematopoietic progenitor cells (Cattoglio et al., 2010). To improve the biosafety profile, self-inactivating vectors (SIN) have been developed. These vectors contain deletions of the regulatory elements in the downstream 3' long

terminal-repeat sequence (LTR), eliminating the transcription of the packaging signal required for vector replication (Zufferey et al., 1998b).

1.7.2 Promoter elements

Strong viral promoters are required for efficient viral induction and propagation. Viral promoters include cytomegalovirus immediate early (CMV-IE) promoter, the simian virus 40 (SV40), rous sarcoma virus long terminal repeat (RSV-LTR), moloney murine leukaemia virus (MLV) LTR and other retroviral LTR promoters. Their use *in vitro* has been widespread however there is considerable evidence that viral promoters are prone to inactivation and silencing *in vitro* and *in vivo*, either via specific down-regulation by interferons (Acsadi et al., 1998, Ghazizadeh et al., 1997) or other additional complex silencing mechanisms (Prasad Alur et al., 2002). The use of eukaryotic promoters, such as the non-tissue specific phosphoglycerate kinase promoter (PGK) or the human elongation factor 1α short promoter (EF1α) may confer a greater advantage in achieving long-term trans-gene expression *in vivo* and have been shown to be reduce the genotoxic risks due to insertional mutagenesis and activation of proto-oncogenes (Zychlinski et al., 2008).

1.7.3 Enhancer Element

Enhancers are among the set of eucaryotic promoter elements that appear to increase transcriptional efficiency in a manner relatively independent of their position and orientation with respect to a nearby gene. The prototype enhancer, the 72 bp tandem repeat of SV40 DNA is located more than 100 nucleotides upstream from the cap site of the early viral genes and was found to be required in cis for efficient gene transcription (Swimmer and Shenk, 1984). In current gene therapy clinical trials the CMV enhancer element is most frequently used as it has been shown to increase transgene expression

level under the control of a variety of different tissue specific promoters in different cell types (Powell et al., 2015).

1.7.4 Cassette

Woodchuck hepatitis virus post-translationally regulated element (WPRE) is an RNA element shown to enhance protein expression (Donello et al., 1998). Insertion of the WPRE in the 3' untranslated region of coding sequences carried by either oncoretroviral or lentiviral vectors substantially increased their levels of expression in a transgene-, promoter- and vector-independent manner (Zufferey et al., 1999). Use of the WPRE in SIN-LV enhances titre levels and ensures stably transmitted vectors with high-level erythroid-specific expression for gene therapy of red cell diseases (Moreau-Gaudry et al., 2001).

1.7 Gene therapy strategies for RDEB

The use of gene therapy for RDEB mitigates the risks associated with HLA-mismatched allogeneic cell transfer. In order to transfer genetic material, gene delivery vehicles such as viral vectors or non-viral transposon systems or phage mediated transfer have been used.

1.7.1 Non-viral vectors

The use of bacteriophage delivery systems have been shown to be a relatively safe method of gene delivery (Larocca and Baird, 2001). The φC31 based integrase vectors have been effectively used and showed correction of RDEB phenotype *in vitro* (Ortiz-Urda et al., 2002a). Primary RDEB epidermal keratinocytes were transfected with a stably integrating transfer vector encoding *COL7A1* cDNA driven by a CMV promoter and a φC31-integrase expression plasmid. Cells were then enriched using blasticidin,

a selective antibiotic, following transfection in order to enrich the transfected cell population. Skin regenerated using these cells displayed stable correction of hallmark RDEB disease features, including C7 protein expression and AF formation. This approach was also then applied to RDEB fibroblasts with impressive results showing that corrected intradermal fibroblasts were able to restore dermal-epidermal adhesion in a *scid/scid* xenograft mouse model (Ortiz-Urda et al., 2003a). In addition, there was a small encroachment of correction beyond the initial site of intradermal administration, perhaps due to overexpression of C7 in engineered fibroblasts. Importantly this study also demonstrated the superiority of autologous gene corrected fibroblasts in terms of C7 delivery compared with normal allogeneic cell (Ortiz-Urda et al., 2003a). More recently polymer mediated technology has been used to transfect RDEB cells with a full length *COL7A1* transcript (Cutlar et al., 2015). Highly branched poly (β-amino ester) HPAE can mediate a 22.3 and 3-4 fold increase in transfection efficiency in keratinocytes with lower cytotoxicity than previous commercial plasmid measures. Further approaches have also included Spliceosome-mediated RNA trans-splicing (SMaRT) which is an RNA-based technology to reprogramme genes for diagnostic and therapeutic purposes. SMaRT protocols have recently been used for in vitro phenotypic correction of a variety of genetic disorders and have been recently been applied to epidermolysis bullosa. Peking et al (2016) have used this system to restore C7 expression in murine keratinocytes. Using SMaRT model systems they were able to design a 5'RNA trans-splicing molecule capable of replacing *COL7A1* exons 1-15. These were transferred to murine skin using minicircle-coated gold particles. The novel non-invasive and pain-free administration of the vector using a gene-gun application is an exciting step forward for in vivo study designs.

1.7.2 γ-retroviral vectors for RDEB

γ-retroviral vectors are the most widely used vehicles in human gene trials (Khavari et al., 2002, Gache et al., 2004a) and have been used to transduce keratinocytes with *COL17A1* (Seitz et al., 1999*), LAMB3* (Vailly et al., 1998), *ITGB4* (Dellambra et al., 2001) as well as *COL7A1*. Most retroviral vectors only have the capacity for 7-8kb and therefore use of these vectors in RDEB had been limited by the large size (9.2kb) of the *COL7A1* cDNA. In order to overcome this challenge Chen *et al* (2006) developed a truncated recombinant type VII mini-collagen containing the intact non-collagenous domains, NC1 and NC2, and part of the central collagenous domain minus a 683-amino acid in-frame deletion (deletion from amino acids 1920 to 2603). (Chen et al., 2000a). Retroviral-mediated transduction of the minigene construct into RDEB keratinocytes (C7 null) resulted in persistent synthesis and secretion of a 230-kDa recombinant mini-collagen VII. However, the consequences and efficiency of truncated proteins has yet to be demonstrated. An alternative method of gene correction could be through the use of trans-splicing technology. Trans-splicing is a gene repair mechanism, using the cell's spliceosome to recombine an endogenous target pre-mRNA and an exogenously delivered RNA molecule called pre trans-splicing molecule (PTM) (Murauer et al., 2011). Gene correction of RDEB patient keratinocytes was achieved by 3 trans-splicing in which intron 64 was specifically targeted, allowing the reduction of 9.2-kb *COL7A1* cDNA to a 3.3-kb transgene. Thus this mechanism could feasibly correct the phenotype of RDEB keratinocytes with mutations downstream of exon 65.

Studies using a canine RDEB model were able to demonstrate efficient transfer the full length 9-kb *COL7A1* cDNA into keratinocytes and achieve correction of RDEB

phenotype *in vitro* (Baldeschi et al., 2003). Full length *COL7A1* cDNA was packaged using two vector backbones LZRS-Col7 and MSCV-Col7 (murine stem cell vector). Transduction with the MSCV-Col7 vector yielded a range of 83–93% positive cells compared with 40-60% using the LZRS-Col7 vector; the highest efficiencies obtained with the retrovirus pseudo-typed with the MLV amphotropic envelope. Gache *et al* (2004) expanded on this further and showed that transplantable fibrin-based skin equivalents made with the transduced RDEB keratinocytes and grafted onto SCID mice generated cohesive and normal stratified epithelia including rapid formation of AFs (Gache et al., 2004a). The same group then was able to transplant the skin equivalents onto two dogs to demonstrate the longevity of the genetic correction. In the first animal, the grafted epidermis firmly adhered to the dermis throughout the 24-month follow-up, which correlated with efficient transduction (100%) of highly clonogenic epithelial cells and sustained transgene expression. In a second dog less efficient (65%) transduction of primary keratinocytes resulted in a loss of the transplanted epidermis and graft blistering 5 months after transplantation (Gache et al., 2011). These disparate findings highlight an important issue of target cell selection and the importance of stem cell selection for autologous skin equivalent grafts incorporating epidermal keratinocytes ie possible inefficient targeting of highly clonogenic keratinocyte stem cells, key in providing the auto-renewal capacity of the grafted tissue.

The use of classical γ-retroviral vectors is limited in clinical trials due to the known risks of insertional mutagenesis. Titeux *et al.* (2010) have been able to demonstrate the use of safer SIN retroviral vectors encoding the full-length *COL7A1* cDNA driven by two alternative human endogenous promoters *COL7A1* or EF1α (Titeux et al., 2010). Using these vectors efficient titres of viral supernatant were generated without

necessitating antibiotic selection. Primary RDEB keratinocytes and fibroblasts were transduced with high efficiencies (30-80% of keratinocytes and 38-70% of fibroblasts) and were able to show long-term restoration of phenotype using human skin equivalents grafted onto nude mice. The titre of their retroviral vector was far superior to the lentiviral equivalent, however the main concern had been the risk of gene and protein rearrangements. This work has then formed the basis of the development of a phase I clinical trial discussed within this thesis.

1.7.3 Lentiviral vectors for RDEB

Concerns regarding the pro-oncogenic potential of γ-retroviral vectors following the SCID-X cases (Hacein-Bey-Abina et al., 2003) then led to further developments and trials of safer lentiviral vectors. As well as the drive for safer constructs, lentiviral vectors have been shown to have greater efficiency for transduction of wild type clonogenic primary keratinocytes (Gagnoux-Palacios et al., 2005), their main advantage being due to the ability of lentiviral vectors to transduce both dividing and non-dividing cells (Naldini et al., 1996b).

RDEB keratinocytes and fibroblasts have been successfully transduced *in vivo* with a SIN lentiviral vector containing full-length *COL7A1* transgene (Chen et al., 2002b). This vector contained a murine LTR based internal promoter designated MND (modified from the murine leukaemia virus) which contained altered methylation target sites and negative control elements within the promoter that have been shown to be resistant to silencing in mouse stem cells in culture and *in vivo.* Gene transfer done at multiplicities of infection (MOI) of 25 achieved >95% efficiency in unselected RDEB cells with no immunostaining detected in the parent RDEB cells. Correction was sustained for up to 5 months *in vitro*. The gene corrected cells were also used to regenerate a human skin equivalent which was then grafted onto mice. C7 expression

at grafted sites was shown to be comparable to normal controls. Woodley *et al* (2003) have also transduced fibroblasts using a SIN-lentiviral vector expressing *COL7A1* cDNA under the control of retroviral-MND promoter demonstrating that the intradermally injected fibroblasts synthesised and secreted C7 for up to 4 months leading to anchoring fibril formation at the DEJ. Furthermore, intradermal administration of 5×10^6 of gene corrected fibroblasts alone could lead to sustained C7 expression, without the need for reconstituted keratinocytes (Woodley et al., 2003).

The same group then developed an alternative *in vivo* strategy administering the lentiviral vector directly into skin (Woodley et al., 2004b). VSV-G-pseudotyped LVs expressing human C7 intradermally were injected into the skin of athymic hair-less mice and also into an RDEB skin equivalent grafted onto a nude mouse. Skin biopsies taken up to 12 weeks after only one administration of LV vector showed sustained C7 expression. Interestingly there was only minimal transduction of epidermal keratinocytes following the direct transdermal approach, presumably due to the large vector particles being blocked by the DEJ proteins. Although this approach is more straightforward than the *ex vivo* strategy, the concerns regarding bio-safety would add regulatory limitations on clinical trials utilising this method.

1.7.4 Autologous cultured skin grafts

In 1975,Rhinewald and Green first published their discovery that epidermal keratinocytes could be cultured *in vitro* (Rheinwald and Green, 1975). Subsequently, the development of tissue culture techniques led to revolutionary therapies for the management of burns victims (Gallico 1984). Cultured epidermal grafts also have been shown to promote re-epithelialization in some genetic skin diseases associated

with chronic wounds, including epidermolysis bullosa (McGrath et al., 1993, Hill et al., 1992, Roseeuw et al., 1994). The premise of using an autologous cultured skin graft following *ex vivo* gene transfer is fascinating. Thus far, the only successful clinical trial of *ex vivo* cutaneous gene therapy was in a 36 year old male man with generalised intermediate JEB (Mavilio et al., 2006). This form of JEB displays generalized blistering, nail loss and alopecia. Pathogenic mutations occur in *LAMA3*, *LAMB3*, *LAMC2*, and *COL17A1*, encoding for proteins critical for hemidesmosome assembly at the dermal-epidermal junction. The particular individual studied was a compound heterozygote for two mutations in *LAMB3*, encoding the β3 polypeptide of laminin-332. Keratinocytes cultured from an acral skin biopsy were transduced with a Molony leukaemia virus (MLV) retroviral vector carrying full length *LAMB3* cDNA. Finding sufficient holoclones necessary for epidermal graft generation was difficult: ideal cells were only cultivated from the palm, all other biopsy sites showing marked stem cell depletion. Nine epidermal grafts using these genetically corrected cells were surgically grafted onto prepared areas on the upper thigh. Wound beds were prepared using Timedsurgery (electrosurgery) (Guerra et al., 2000). The regenerated epidermis healed well and remained blister free with no evidence of graft infection or adverse immune response. Furthermore, quantitative PCR analysis and immunofluorescence studies up to 12 months following surgery showed full-length laminin-332 protein expression as well as continued transgene expression. Follow-up 7 years post-grafting confirmed that the regenerated skin still expressed normal amounts of laminin-332 with phenotypically resilient, non-blistered grafted skin (De Rosa et al., 2014) . At this stage further patient recruitment was halted in order for the cell therapy facility in Modena to conform to the 2007 EU directive 1394 imposing Good Manufacturing Practices for advanced Medicinal Products. There were also concerns regarding the

genotoxic potential of classical MLV- retroviral vectors. Insertional activation of a T cell proto-oncogene has been correlated with the occurrence of lymphoproliferative disorders in gene therapy trials of X-linked severe combined immunodeficiency (X-SCID) (Hacein-Bey-Abina et al., 2003 and Hacein-Bey-Abina et al., 2008) and Wiscott-Aldrich syndrome (WAS) (Aiuti et al., 2012 and Boztug et al., 2010). The trial was suspended pending further development of safer SIN vectors in order to minimize the possible risks of insertional mutagenesis. One further patient with JEB has been treated in Austria using the same vector for cell transduction as well as the same grafting methodology (Murauer et al., 2015). Five skin sheets each measuring 5 x 7 cm were grafted onto the patients' thighs. Data concerning the 1-year post transplantation follow-up are yet to be published.

More recently, Siprashivili *et al* (2014) have now provided the first reports of the first subject enrolled for Phase I trial of genetically corrected autologous epidermal keratinocyte sheet for adults with RDEB trial in Stanford, USA (NCT01263379). Their study uses epidermis regenerated from retrovirally transduced autologous keratinocytes, LZRSE-*COL7A1* Engineered Autologous Epidermal Sheets (LEAES). The first subject was a 23 year old man with severe RDEB with truncated C7 expression and sparse rudimentary AFs visible on electron microscopy. Six ~35cm^2 autologous epidermal sheet grafts were grafted onto prepared wound beds. Thirty days following grafting the skin was clinically normal and on biopsy robust C7 expression was detectable (Siprashvili et al., 2014). This study is currently still recruiting and aiming to treat up to 5 patients.

1.7.5 Genome editing

Genetic manipulation to rescue disease phenotype has now progressed to include gene editing, using innovative technologies such as zinc finger nucleases (ZFN), transcription activator like effector nuclease (TALEN), the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease systems and meganucleases. The general mechanism aims to correct a mutation by providing a template for repair via homologous recombination (HR). Engineered nucleases are used as "molecular scissors" to generate a double stranded DNA break (DSB) at a specific location within the genome, subsequently promoting correction by HR with an exogenous DNA repair matrix (Urnov et al., 2005). Over the past 5 years, the potential of such gene editing strategies to correct inherited disorders at a pre-clinical level, both in animal models and human cells, has been demonstrated (Genovese et al., 2014, Lombardo et al., 2007). Hockemeyer *et al* were able to demonstrate the use of TALEN nucleases in genome editing of human iPSC cells (Hockemeyer et al., 2011). These technologies have been optimized to correct mutations in the genes for keratin 14 (Wally et al., 2010), collagen XVII, plectin (Wally et al., 2008) and collagen VII (Osborn et al., 2013b) in vitro.

Izmiryan *et al* (2016) have recently demonstrated the feasibility of correcting *COL7A1* mutations using meganucleases (MNs) to mediate HR (Izmiryan et al., 2016). They used integration-deficient lentiviral vectors for delivery of meganucleases, the expression of which was controlled by EF1 promoter. *COL7A1* correction in primary RDEB-K and RDEB-F carrying a homozygous null mutation (c.189delG; p.Lys64Trpfs*40) in exon 2 was achieved and no off-target activity was detected, although gene correction efficiency was low (<10% transcript correction). In addition to the plethora of nuclease technologies that have developed over the last 5 years,

AAV viral vectors previously used within gene therapy trials have been used as part of gene editing strategies. The transgene capacity is sufficient to contain ZFN monomers as well as donor constructs, and this approach has been used successfully, one of the main advantages being the minimisation of delivered viral particles to the cell which have been shown to cause significant toxicity (Ellis et al., 2013). Sebastiano et al (2014) have also used recombigenic species of adeno-associated virus to genetically edit *COL7A1* mutations (Sebastiano et al., 2014). Two patient derived RDEB cell lines with mutations in exons 2 and 3 of the *COL7A1* locus were targeted by an AAV mediated system that spanned 6 exons and contained 1.4 kb targeting arms on either side of a central puromycin selection cassette. Southern blotting and Sanger sequencing confirmed an absence of random integration events within the *COL7A1* locus. The corrected cell types in these experiments were iPSCs derived from RDEB keratinocytes and fibroblasts, which were then transformed into corrected keratinocytes. These were used to create autologous epithelial grafts with normal C7 composition. This approach has also been demonstrated in cells derived from a patient with generalised severe JEB. Melo *et al* (2014) used recombinant adenoassociated virus mediated HR to correct mutations at the *LAMA3* locus in primary keratinocytes (Melo et al., 2014). Critical to the development of the use of AAV platforms would be to maximise rates of homology-directed repair by using nucleases to generate DNA breaks (Miller et al., 2003). Thus far there have been no proposed phase 1 clinical trials for these editing platforms, and further work needs to be done to improve the correction efficiency, compare the technologies within the same disease and ensure sufficient safety data necessary from a regulatory aspect.

1.7.6 Revertant mosaicism – "natural gene therapy"

Revertant mosaicism (RM) refers to the coexistence of cells in one individual carrying germline mutations together with cells in which the inherited, disease-causing mutation is corrected by a spontaneous genetic event resulting in partial or complete restoration of phenotype. These corrected cells are termed "revertant". Several mechanisms can account for RM, including a reverse point mutation, crossing-over, gene conversion, and a second-site mutation (Jonkman et al., 1997). The first description of RM in human skin was reported by Jonkman *et al* (1997) in a 28 year old woman with autosomal recessive JEB and a compound heterozygous mutation in *COL17A1* (Jonkman et al., 1997). The mechanism behind this gene conversion was reciprocal transfer of part of the parental allele leading to loss of heterozygosity in the patches of normal appearing skin. The most common mechanism for reversion has recently been suggested to be mitotic recombination. Kiritsi et al (2014) described one of the largest cohorts of DEB patients with revertant mosaicism and determined that back mutation/mitotic recombination to be the underlying mechanism in up to 70% of cases and second site mutations affecting splicing in up to 30% (Kiritsi et al., 2014). RM has been reported to occur most commonly in JEB *COL17A1* (Pasmooij et al., 2007), but has also been found in the skin of patients with mutations in *KRT14* (Schuilenga-Hut et al., 2002), *LAMB3* (Pasmooji 2005), *FERMT1* (Lai-Cheong et al., 2012, Kiritsi et al., 2012) as well as *COL7A1* (Almaani et al., 2010, Pasmooij et al., 2010, van den Akker et al., 2015). Although C7 is synthesized and secreted by both keratinocytes and fibroblasts, evidence for reversion has only been found in keratinocytes. This mechanism offers a unique opportunity for the use of revertant cells in autologous cell based therapies for RDEB, and yet attempts for culture of revertant keratinocytes and subsequent grafting have been unsuccessful (Gostynski et

al., 2009). Gostynski *et al* attempted this technique in a patient with JEB, however a biopsy taken from a patch of skin with ~30% reversion yielded a graft with < 3% corrected keratinocytes, raising possible issues of maintaining revertant cells in culture as well as the risks of mutant stem cell colonisation within the graft. In contrast the use of punch grafting revertant cells has shown stable and long-lasting reversion of phenotype and genotype in a patient with JEB, in this case treating a patient's chronic ulcers (Gostynski et al., 2014).

1.7.7 Induced pluripotent stem cells

In 2006, Takahashi and Yamanaka first demonstrated that stem cells with properties similar to those of embryonic stem cells could be generated from mouse fibroblasts by simultaneously introducing four transcription factors (c-MYC, SOX2, OCT4 and KLF4) (Takahashi and Yamanaka, 2006), work that was subsequently extended to human fibroblasts (Takahashi et al., 2007). These stem cells were designated induced pluripotent stem cells (iPS cells or iPSCs). This exciting discovery demonstrated that almost all differentiated somatic cells had the capability to differentiate to a pluripotent state, albeit at different efficiencies. Bilusova et al (2011) were able to demonstrate directed differentiation of mouse iPS cells in culture into a multipotent keratinocyte lineage capable of forming a fully differentiated epidermis, hair follicles, and sebaceous glands in a reconstitutive *in vivo* environment (Bilousova et al., 2011). Simultaneously Tolar *et al* (Tolar et al., 2011) were able to demonstrate differentiation of gene corrected RDEB cells into corresponding iPS cells was similar to that observed for wild-type iPS cells. Interestingly, these investigators also observed differentiation of these cells into hematopoietic lineages, which suggests that such cells could be used to generate autologous hematopoietic cells for grafting. Recently iPS cells have been generated from revertant keratinocytes of a junctional EB patient with compound

heterozygous mutation in *COL17A1* (Umegaki-Arao et al., 2014). Revertant iPSC keratinocytes were then used to create *in vitro* three-dimensional skin equivalents and used to reconstitute human skin *in vivo* in mice, expressing full length C17. Proof of principle studies have also demonstrated the safety and efficacy of iPSCs for RDEB in murine models (Wenzel et al., 2014) as well as *in vitro* (Tolar et al., 2014). Sebastiano *et al* have used adenovirus-associated viral genome editing to generate C7 corrected iPSC banks to derive keratinocytes using GMP facilities and procedures. Cells were produced with minimal heterogeneity, and these cells secreted wild-type type C7 resulting in stratified epidermis *in vitro* in organotypic cultures and *in vivo* in mice (Sebastiano et al., 2014).

The use of iPSCs taken from revertant skin could be an ideal therapeutic strategy, as this would obviate some of the technical difficulties and risks associated with gene therapy or gene editing. Tolar *et al* were able to demonstrate that revertant RDEB keratinocytes expressing functional C7 can be reprogrammed into iPSCs and that self-corrected RDEB iPSCs can be induced to differentiate into either epidermal or hematopoietic cell populations (Tolar et al., 2014). However spontaneous reversion remains rare, with only a few patients with RDEB confirmed to have patches of RM and therefore this does not necessarily offer a viable and universally applicable therapeutic option.

Table 1.2 Summary of gene correction strategies in inherited skin disorders

Method	Target gene	Condition	Target cell	Comments	Reference
Self-inactivating lentiviral vector	*COL7A1*	RDEB	In vivo	In vivo inoculation of skin equivalents grafted onto mice, C7 expression up to 3 months	(Woodley et al., 2004b)
Self-inactivating lentiviral vector	*COL7A1*	RDEB	Fibroblast	Gene corrected fibroblasts injected into murine skin able to produce C7 at basement membrane zone	(Woodley et al., 2003)
Gamma retroviral vector	*COL7A1*	RDEB	Keratinocyte	Construction of skin equivalents using classical retroviral gene transfer; transduction efficiency 40%	(Gache et al., 2004b)
Self-inactivating gamma retroviral vector	*COL7A1*	RDEB	Keratinocyte + Fibroblast	Generation of human skin equivalents showing in vivo functional correction of phenotype; C7 expression of recombinant C7 ranging between 30-80% keratinocytes and 40-70% fibroblasts	(Titeux et al., 2010)
Retroviral-vector	*COL7A1*	RDEB	Keratinocyte	Genetic correction of both dog RDEB and human primary RDEB cells	(Baldeschi et al., 2003)
φC31 integrase based plasmid	*COL7A1*	RDEB	Keratinocyte	Non-viral approach into primary RDEB cells; regenerated skin (40% efficiency); stable correction required short-term drug selection and enrichment	(Ortiz-Urda et al., 2002b)
3' pre-transplicing molecule	*COL7A1*	RDEB	Keratinocyte	Trans-splicing used to reduce size of *COL7A1* transcript and therefore risk of genetic rearrangement	(Murauer et al., 2010)
φC31 integrase based plasmid	*LAMB3*	JEB	Keratinocyte	Non-viral approach showing corrected hemidesmosome formation in JEB primary cells	(Ortiz-Urda et al., 2003b)
Transposase (sleeping beauty)	*LAMB3*	JEB	Keratinocyte	Sleeping beauty transposable element used to integrate LAMB3 cDNA into epidermal cells in 6 JEB patient primary cells; transfected cells enriched using drug selection.	(Ortiz-Urda et al., 2002b)
PAC (P1-based artificial chromosome clone)	*COL7A1*	RDEB	Keratinocyte	Gene transfer using micro-injection of entire human locus *COL7A1* in a P1 derived artificial chromosome	(Mecklenbeck et al., 2002)

TALEN gene editing	*COL7A1*	RDEB	Fibroblasts	Transcription activator-like endonucleases (TALEN) gene editing of RDEB fibroblasts subsequently reprogrammed into iPS cells (induced pluripotent stem cells)	(Osborn et al., 2013a)
Gamma retroviral vector	*COL17A1*	JEB	Keratinocyte	Restoration of full-length BP180 protein expression in primary keratinocytes then used to regenerate human skin on immunodeficient mice	(Seitz et al., 1999)
Gamma retroviral vector	*LAMB3*	JEB	Keratinocyte	Only case of successful gene transfer in an inherited skin disorder in an adult patient with non-herlitz junctional EB; restored phenotype and laminin 332 expression 7 years post graft	(Mavilio et al., 2006)
Self-inactivating lentiviral vector	*LAMB3*	JEB	Keratinocyte	Pre-clinical study using potentially safer lentiviral (LV) vector	(Di Nunzio et al., 2008)
Gamma retroviral vector	*XPC*	Xeroderma pigmentosum	Keratinocyte + Fibroblast	Functional in vitro correction of XPC keratinocytes	(Arnaudeau-Begard et al., 2003)
Adenoviral vector	*XPA*	Xeroderma pigmentosum	In vivo	In vivo inoculation of recombinant AV vector in UV irradiated murine skin	(Marchetto et al., 2004)
Self-inactivating lentiviral vector	*XPA/XPC/XPD*	Xeroderma pigmentosum	Fibroblast	Efficient SIN LV vector able to correct cellular phenotype in XP	(Marchetto et al., 2006)
Gamma retroviral vector	*XPC*	Xeroderma pigmentosum	Keratinocyte	MLV derived vector able to transduce primary cells and maintain XPC expression in up to 130 population doublings	(Warrick et al., 2012)
TALEN gene editing	*XPC*	Xeroderma pigmentosum	Fibroblast cell line	Successful targeted gene correction of XPC cell line	(Dupuy et al., 2013)
Self-inactivating lentiviral vector	*LEKTI*	Netherton syndrome	Keratinocyte	Pre-clinical work demonstrating full length *LEKTI* expression in organotypic cultures grafted onto mice. Currently recruiting for phase I clinical trial	(Di et al., 2011)
Retroviral vector	*STS*	X-linked ichthyosis	Keratinocyte	Restoration of enzymatic activity in cultured primary keratinocytes deficient in steroid sulfatase	(Freiberg et al., 1997)
siRNA	*KRT5*	EB Simplex	Keratinocyte	Pre-clinical study, specific knockdown of mutant keratin 5 protein	(Atkinson et al., 2011)

siRNA	*K6a mRNA*	Pachyonychia Congenita	Keratinocyte	Phase 1b trial injecting TD101 siRNA locally for plantar keratoderma in PC	(Leachman et al., 2010)
Direct injection of plasmid	*TGM1*	LI	Keratinocyte	Non-uniform restoration of gene expression on regenerated LI skin	(Choate et al., 1996)

AV= adenoviral vector, JEB = Junctional epidermolysis bullosa, LI = Lamellar ichthyosis, LV = lentiviral vector, MLV = Murine leukaemia virus, RDEB = Recessive dystrophic epidermolysis bullosa, SIN = self-inactivating, UV = ultraviolet.

1.8 Target cells for correction

In order for sustained genetic correction in the skin it is critical to target the slow dividing stem cell population. The definition relies on the property of unlimited or prolonged self-renewal and multipotency of stem cell capacity to produce at least one type of differentiated descendant. These stem cells are normally slow-cycling *in vivo* but possess high proliferative potential which can be triggered in response to tissue injury or to certain growth stimuli.

1.8.1 Epidermal stem cells

The epidermis is continually renewing and replaced every 18 days in adults (Epstein and Maibach, 1965). In order to accomplish this, the epidermis relies on epithelial stem cells. These cells are extremely slow-cycling and may undergo symmetrical division to form a few transit amplifying cells capable of limited further divisions. One hypothesis is of an epidermal proliferating unit (EPU) consisting of a single epidermal stem cell surrounded by transit amplifying cell progeny at the base of terminally differentiated keratinocytes (Potten and Morris, 1988). Slow cycling epidermal cells have been studied *in vitro* via so called label-retaining cells (LRCs), owing to their ability to retain radioactive thymidine incorporated into their DNA for prolonged periods of time after labelling (Mackenzie and Bickenbach, 1985). Using these techniques, epidermal stem cells have been shown to reside in 3 main areas: the first was localized to the inter-follicular basal layer of epidermis; the second was shown to reside in the bulge area of hair follicles and a third population was localized to the upper isthmus, a region between the bulge and the sebaceous gland. Keratinocytes grown in culture have been divided into three subtypes according to proliferative

capability: holoclones, meroclones and paraclones (Barrandon and Green, 1987). Holoclones have the greatest reproductive potential as under standard conditions, fewer than 5% of the colonies formed by the cells of a holoclone abort and terminally differentiate (Barrandon and Green, 1987). Indeed, a single holoclone has been shown to have the capability to generate the entire human epidermis (Rochat et al., 1994). (Jones Ph, 1993, Pellegrini et al., 1999). Subsequent studies have shown that epidermal stem cells firmly adhere to basement membrane and represent a minor subpopulation (~2–7% of basal keratinocytes) of relatively quiescent cells (Bickenbach, 1981, Li et al., 1998). The first progeny of stem cell division, transient amplifying (TA) cells, have a limited proliferative potential, yet share many molecular markers with their parent cell population (Jones Ph, 1993). Epidermal stem cells are also capable of asymmetrical cell division and forming daughter cells committed to terminal differentiation without the need for TA cells at least in murine epidermis (Blanpain and Fuchs, 2009).

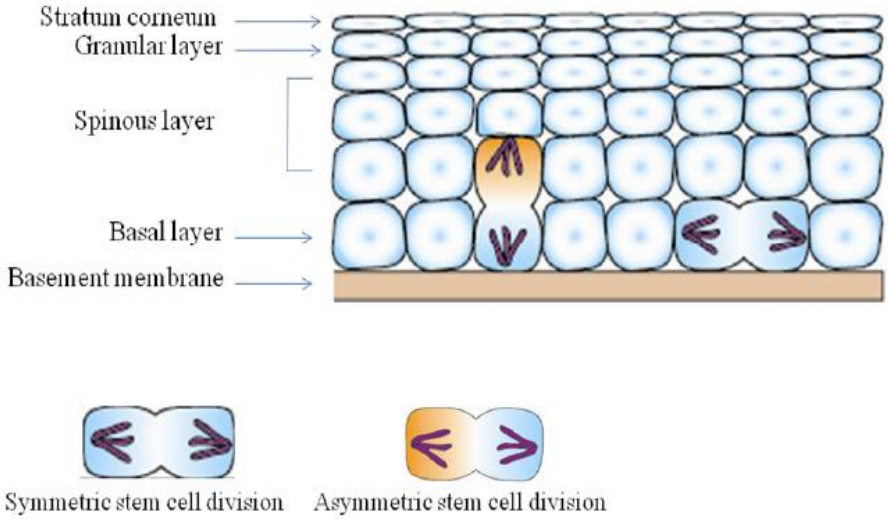


Figure 1-10 Schematic demonstrating symmetric and asymmetric stem cell division (adapted from Fuchs and Chen 2013)

The asymmetrical cell division model to explain the ability of stem cells to self- renew in the long-term. At the end of anaphase, one daughter has received an excess of stem cell factors and remains undifferentiated, whereas the second daughter receives lineage commitment factors and embarks on a differentiated pathway.

The challenge lies in ready identification of cell types as an agreed panel of epidermal stem cell surface markers has not yet been characterised. There are multiple approaches available in order to identify and isolate a pure EpSC population, including combined use of cell kinetic analysis and cell sorting (FACS) *in vivo* (Tani et al., 2000, Li et al., 1998). Keratinocytes with stem-like properties have been enriched using markers such as β1 integrin (Jones and Watt, 1993), α6 integrin (Kaur and Li, 2000), the nuclear transcription factor p63 (Pellegrini et al., 2001), LRIG-1 (Jensen and Watt, 2006) amongst many others. In addition, classical developmental signalling pathways such as Notch, WNT, BMPs, Noggin, and SHH have all been reported to play important roles in maintaining adult stem cell niches (Fuchs, 2008). However, the majority of advances in stem cell biology and the development of these panels of

markers have been accomplished in mouse models. How far this can be applied to human cell biology is unclear.

1.8.2 Dermal stem cells

The adult dermis contains several types of progenitor cells, including skin-derived precursors (SKPs), neural crest-derived stem cells (NCSCs), melanoblasts, perivascular cells (PCs), endothelial progenitors (EPs), and adipose-derived stem cells (ADSCs) (Feisst et al., 2014, Gimble et al., 2007, Fernandes et al., 2004, Murga et al., 2004, Chang et al., 2014). The main cellular component of dermal tissue are dermal fibroblasts and the heterogeneity of fibroblasts is only recently being explored. Chen *et al* have shown that nestin-negative, vimentin-positive fibroblasts may represent a novel type of multipotent adult stem cells in human dermis (Chen et al., 2007). Kuroda *et al* further delineated a stem cell population named multilineage-differentiating stress-enduring (MUSE) cells in human dermal fibroblasts that are characterized by stress tolerance, expression of pluripotency markers, self-renewal, and the ability to differentiate into endodermal-, mesodermal-, and ectodermal-lineage cells from a single cell (Kuroda et al., 2010). These cells are also a primary cell source for induced pluripotent stem cell generation (iPS cell) in human fibroblasts.

Recently lineage tracing has shown that dermal fibroblasts arise from a multipotent MSC cell population expressing PDGFRα, delta like homolog-1 (Dlk1) and LRG1 (Driskell et al., 2013). Upon differentiation, the fibroblasts begin to express different markers distinguishing common progenitor fibroblasts from specific fibroblasts within the papillary dermis (BLIMP1, LRG1) and those within the reticular dermis (Dlk1) (Driskell et al., 2013, Sorrell and Caplan, 2004). The heterogeneous cell lineages may also have differing functional capacity. Lineage studies have shown that the initial

phase of dermal repair occurring during wound healing is mediated by lower lineage fibroblasts, expressing myofibroblast markers such as SMA (Driskell et al., 2013). These cells secrete large amounts of ECM proteins including collagens. Therefore, transplantation of fibroblast lineages from the upper papillary dermis may have be more beneficial for promoting scar free wound healing as they secrete less fibrillar collagen than lower dermal fibroblasts. In contrast transplantation of fibroblasts derived from lower reticular dermis that can differentiate into hypodermal adipocytes may be of benefit for surgical reconstructions requiring fibrofatty scar tissue (e.g. breast reconstruction).

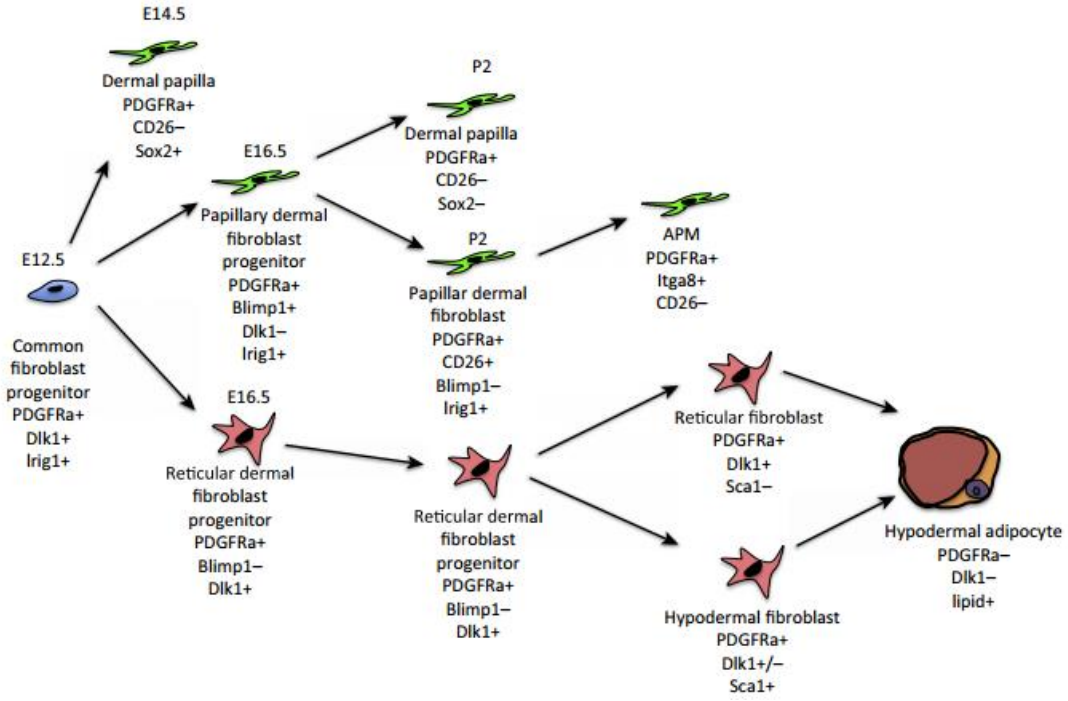


Figure 1-11 Lineage relationships of fibroblasts determined on murine skin (Driskell and Watt 2015).

Fibroblasts arise from a common progenitor and progressively differentiate into upper fibroblast lineages and lower fibroblast lineages. A cluster of fibroblasts originating from the dermal papillae form the smooth muscle known as the arrector pili muscle (APM) in response to signals from the adjacent epidermal stem cells within the hair bulge. Subsets of reticular dermal fibroblasts can be differentiated into adipocytes.

1.8.3 Bone marrow derived stem cells

Bone marrow stem cells (BMSCs) demonstrate plasticity in being able to contribute to mesodermal (e.g. muscle), endodermal (e.g. liver) and ectodermal (e.g. neural tissue, skin) structures (Grove et al., 2004). Indeed, BM cells contribute a substantial proportion of cells in the skin, both inflammatory and non-inflammatory, that have roles in skin development, homeostasis, repair and regeneration. Some sub-populations of the BM cells can differentiate into skin epithelial cells (keratinocytes) (Badiavas et al., 2003, Borue et al., 2004, Fathke et al., 2004b) and also provide fibroblast-like cells to the skin dermis, although their precise nature is not fully understood. Bone marrow derived stem cells can be derived from either haematopoietic or mesenchymal lineages. Bone marrow derived mesenchymal stromal cells (BM-MSC) are a heterogeneous population, and within that group is a cell type that has been shown to home to wounded skin in murine RDEB models. Tamai *et al*. (2011) showed that the BM-derived MSCs contained a sub-population of epithelial progenitors contributing to the restoration and maintenance of the epidermis (Tamai et al., 2011a). These cells were non-haematopoietic MSCs, with cell sorting determining that they were c-kit negative, lineage (Lin)- negative and platelet derived growth factor receptor alpha (PDGFRα) – positive. The level of this population of MSCs in RDEB bone marrow is unknown.

1.9 Hypothesis and aims of thesis

The development of gene therapy clinical trials for RDEB represents an important milestone in the pursuit of a cure for this devastating disease. There are numerous challenges in the journey to delivering gene therapies into human subjects. This

project aims to develop an *ex vivo* gene therapy approach to deliver a permanent treatment locally in RDEB skin. Specifically:

I. To design and implement a preselection study to ensure appropriate patients are treated with a genetically corrected skin equivalent graft.

II. To provide further rationale for the use of locally delivered gene corrected fibroblasts into RDEB skin.

III. To design a phase I clinical trial of intradermal gene corrected fibroblasts in RDEB.

IV. To identify potential obstacles and challenges in the design, development and implementation of the above studies.

Chapter 2

Materials and Methods

2.1 Research Ethics

Study specific regulatory approvals are mentioned in the relevant chapters. The preliminary studies were performed using research ethics approval by Guy's Research Ethics Committee, Guy's and St Thomas' NHS Trust was used (Characterisation of molecular and structural skin abnormalities in inherited skin disorders, Ref: 07/H0802/104).

2.2 Laboratory techniques

2.2.1 Cell Culture

2.2.1.1 Transport and isolation of primary keratinocytes from skin biopsy

Skin punch biopsy specimens were kept in cell culture medium. The cell culture medium used for transport of skin biopsies constituted a solution of 90% Dulbecco's Modified Eagle Medium, DMEM, (Gibco®, Life Technologies Ltd, Paisley, UK) and 10% standard Foetal Bovine Serum, FBS, (Gibco®, UK) with the addition of antibiotic-antimycotic 100x (Gibco®, UK) and stored at 4ºC for a maximum of 24 hours. Each specimen was trimmed to remove adipose tissue using a sterile scalpel and then incubated in Dispase I protease (Sigma Aldrich Company Ltd, Dorset, England) for at least 12 hours at 4 degrees. This ensured cleavage of the epidermis from the dermis. The epidermis was then minced into 1mm pieces using a sterile scalpel on a plastic tray and then immersed in 10ml 0.05% trypsin/EDTA (Sigma Aldrich, UK). This was incubated at 37ºC for 30 minutes and agitated every 5-10 minutes. Trypsin activity was neutralised with an equivalent volume of media

containing serum and the contents were then filtered through a 100um cell strainer into a sterile falcon tube. The solution was then centrifuged at 1000 rpm for 5 minutes and the pellet resuspended in Epilife® medium (Life Technologies, UK) and plated onto a p60 petri dish which had been previously coated with the Epilife® coating matrix. In cases when samples were to be plated onto 3T3 irradiated feeders, the media used was RM+.

2.2.1.2 Maintenance and passage of keratinocytes

Primary cell cultures were maintained by changing the growth media three times a week. When culture dishes reached 80% confluence, the cells were passaged. A single cell suspension was obtained following incubation for twenty minutes in TrypLE Express (Life Technologies Ltd, UK). An equivalent volume of culture medium containing 10% FBS was added to neutralise trypsin activity and the mixture was centrifuged at 1600 RPM for 7 minutes. The filtrate was discarded by gentle aspiration and the cell pellet re-suspended in fresh media before re-plating into a new petri dish or flask using a ratio 1:4.

2.2.1.3 Isolation of primary fibroblasts from skin biopsy using explant method

After separation of the dermis and epidermis in dispase as shown above, the dermis was placed in sterile cell culture grade Phosphate Buffered Saline (Life Technologies Ltd, UK). Using sterile forceps and scalpel, the dermis was minced into $1mm^2$ pieces. Each piece was dried for 10 minutes and placed directly to a 100mm petri dish. Up to 15 pieces were placed into each dish. Then 12-15 mls of DMEM with 10% FBS was added as well as 1/100 of this volume of 10,000 units penicillin, 10,000 g streptomycin and 25g amphotericin B / ml and stored in a 37ºC 5% CO2 incubator. The cell medium

was replaced with fresh medium every 5 days until growth was seen at 14 days. Once colonies formed, the fibroblasts would be trypsinised and transferred to a new dish.

2.2.1.4 Isolation of primary fibroblasts from skin biopsy using digestion method

After separation of the dermis and epidermis in dispase as shown above, the dermis was placed in sterile PBS. Using sterile forceps and scalpel, the dermis was minced into $1mm^2$ pieces. The minced pieces were then placed in a 15 mls falcon with 2mls of collagenase IV (Life Technologies, UK) and incubated at 37 degrees for at least 4 hours agitating every 20 minutes. Following digestion, the solution was centrifuged at 200G for 10 minutes and the supernatant removed carefully and re-suspended in 3 mls cell culture media and plated in a p60 dish.

2.2.1.5 Cryopreservation and recovery of cells

A cell suspension was obtained and re-suspended in 90%FBS and 10% Dimethylsulphoxide (Fisher Scientific, Leicestershire, UK) in 1.2ml sterile cryovials. The cells were cooled slowly, initially at 80◦ C overnight and then transferred to a liquid nitrogen dewar for long term storage. Cells were recovered by thawing in a 37ºC water bath and then re-suspended in warmed media. The mixture was centrifuged at 1000 RPM for 5 minutes and re-suspended in fresh culture media and re-plated in a dish or flask.

2.2.1.6 Preparation of 3T3's

Frozen vials of 3T3-J2 cells (donated from the Dr Qasim's laboratory, Institute of Child Health, London, UK) were used to generate fresh stocks. These cells were grown in DMEM medium with 10% FBS into T150 flasks. These cells were routinely split two to three times a week in 1:5 dilutions. Cells were trypsinised using 0.05%

trypsin in EDTA, neutralised by 10% FBC/DMEM and then spun the cells down for 5 minutes at 1,000 RPM. Cells were re-suspended in RM^+ and irradiated with 6,000 rads for 20 minutes using a γ-irradiator. Irradiated cells were counted and plated on a fresh flask or dish at the appropriate density. The keratinocytes were then inoculated after at least 2 hours.

2.2.1.7 Sterile culture technique

To prevent contamination of cultured cells by infection, the cells were maintained in a good laboratory practice (GLP) facility and cultured in dedicated class 2 hoods. All surfaces were carefully disinfected with Virkon (Antec International, Suffolk, UK); disposable sterile equipment was used and solutions containing antibiotics prior to use

2.2.1.8 Transduction with viral vector

All procedures involving the use of viral vector were performed in a designated retroviral laboratory tissue culture suite at the Institute of Child Health. Following titration of viral titre, transduction was planned aiming for 1-3 copies per cell, correlating with MOI 3-10%. MOI was calculated using the formula MOI = (Viral titre/1000) x volume viral supernatant (ul)/ Cell number. Cells were then seeded at required density 24 hours before incubation with required viral supernatant. Cell media was then aliquoted to be added to each flask or well. At this point, the vector aliquots were thawed quickly, pooled and then added to the media. Once added, the media was then placed in the well or flask and incubated for a minimum of six hours. The following day, the media was changed. For double transduction, the above process was repeated.

2.2.2 Immunohistochemistry

2.2.2.1 Immunofluorescent staining of skin tissue specimens for C7

Skin samples were cryo-embedded with optimum cutting temperature (OCT) compound (VWR International, Lutterworth, Leicestershire, UK) and cut into 5µm thick sections using Cryostat Bright OTF 5000 (Jencons Scientific, East Grinstead, West Sussex, UK) at -28 C. Normal human skin was used as the positive control and the negative control was normal skin without primary antibody incubation. Frozen sections were air-dried at room temperature for 15 minutes and outlined with a liquid blocking PAP pen (Vector laboratories, California, USA) and then fixed with 4% PFA for 20 minutes. All slides were then washed in PBS for 5 minutes and then permeabilised with Triton X 0.1% for 15 minutes. Following a further 5 minute PBS wash, the sections were blocked with blocking buffer containing 10% goat serum (Sigma-Aldrich, UK) with 0.1% bovine serum albumin (Sigma-Aldrich, UK) diluted in PBS and 0.1% Tween®20 (Sigma-Aldrich, UK). Primary and secondary antibodies were diluted in blocking buffer and incubated for 20 minutes on ice. At all times the secondary antibody was stored in the dark. Following blocking, the sections were incubated with the primary antibody at 4 degrees overnight. The slides were then washed in PBS three times for 5 minutes each. The appropriate secondary antibody diluted in blocking buffer was then added and incubated for 30 minutes at room temperature in the dark. Sections were washed in PBS for 5 minutes and then lastly rinsed in distilled water for 5 minutes. Once air-dry, they were then mounted with DAPI (Prolong Gold Anti-fade) (Life Technologies) and a cover glass applied. The sections were then left overnight to set at room temperature in the dark. For short term storage the sections were kept at 4 degrees and for long term at -80 degrees.

Dilution and selection of primary antibodies to C7 were optimised and all immunohistochemistry illustrated is using the LH 7.2 mouse monoclonal antibody to C7. Polyclonal antibodies obtained from commercial suppliers as well as those prepared by another research groups (courtesy of Prof M. Chen) are illustrated below.

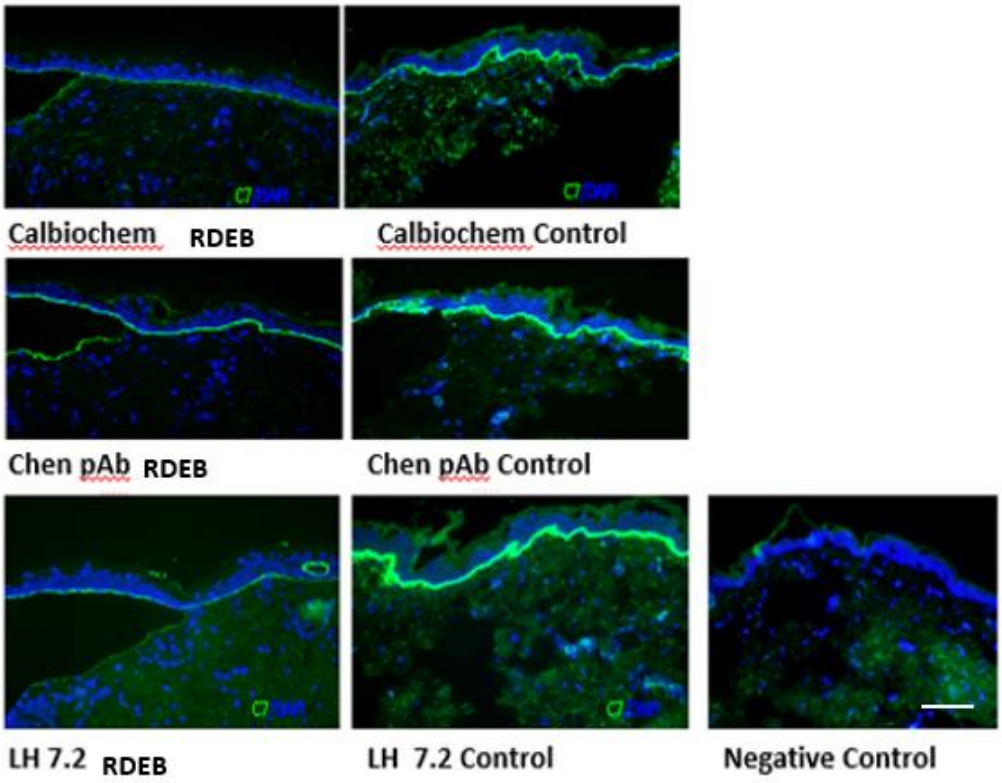


Figure 2-1 Immunohistochemistry using three different antibodies to C7, All 1:200 dilution (see Table 2.1)

Optimisation of the primary anti C7 antibody showed suitable staining with all three anitbodies; LH7.2 mouse monoclonal, Calbiochem rabbit polyclonal and a rabbit polyclonal antibody purified and manufactured courtesy of Professor Mei Chen. Scale bar = 100 µm.

2.2.2.2 Immunofluorescent staining of cultured keratinocytes and fibroblasts for C7.

Coverslips measuring 60μm were sterilised and soaked in culture media for 5 minutes. They were then dried and placed in a 24 well tissue culture plate. Cultured cells were trypsinised, counted and plated in each well at a density of 1×10^5 per cover slip. Cells were cultured for 24-48 hours and then the media was removed and the slips washed with PBS three times for 5 minutes and then fixed with 4% Paraformaldehyde solution for 20 minutes. All coverslips were then washed in PBS for 5 minutes and then permeabilised with Triton X-100 0.1% (Sigma-Aldrich) for 15 minutes. Following a further 5 minute PBS wash, the cover slips were blocked with blocking buffer containing 3% Foetal Bovine Serum diluted in PBS for one hour. Primary and secondary antibodies were diluted in blocking buffer and incubated for 20 minutes on ice. At all times the secondary antibody was stored in the dark. Following blocking, the slips were incubated with the primary antibody at 4ºC overnight. The slides were then washed in PBS three times for 5 minutes each. The appropriate secondary antibody diluted in blocking buffer was then added and incubated for 30 minutes at room temperature in the dark. Sections were washed in PBS for 5 minutes and then lastly rinsed in distilled water for 5 minutes. One drop of Prolong® Gold antifade reagent (Invitrogen) containing 4'6-diamidino-2-phenylindole (DAPI) was added to simultaneously mount and counterstain the nuclei. Prolong Gold preserves the fluorescent signal and DAPI binds to the region of double-stranded DNA rich in A and T bases.

Table 2.1 Antibodies used for immunohistochemistry and immunocytochemistry

Primary Antibody	Secondary Antibody
Mouse monoclonal antibody type VII collagen LH7.2 (Chemicon Europe Hampshire, UK)	Goat anti mouse conjugated to AlexaFluor 488 (Invitrogen); 1:200
Rabbit polyclonal antibody type VII collagen "Chen" (kindly donated from Professor M. Chen, Stanford)	Goat anti-rabbit conjugated to AlexaFluor 488 (Invitrogen); 1:200
Rabbit polyclonal antibody type VII collagen 234192 (Calbiochem, San Diego, USA)	Goat anti-rabbit conjugated to AlexaFluor 488 (Invitrogen); 1:200

2.2.2.3 Quantification of immunofluorescence signals

Quantification of immunofluorescence data was carried out by counting cells showing a positive immunofluorescence signal for C7 in three independent fields containing 100 cells. Data are presented as percentage of cell numbers in relation to the total number of DAPI positive cells. Values are presented as mean +/- standard deviation. Unpaired two-tailed Students' t-test was used to analyse data for statistical significance. Graphs and analysis were generated with GraphPad Prism (Version 6) Software.

2.2.3 Protein analysis

2.2.3.1 Cell protein extraction

Protein was extracted from cultured cells. At least one T25 flask of keratinocytes and one T75 flask of fibroblasts was necessary for appropriate protein extraction. When cells were at 70-80% confluence the culture media was removed and cells washed with fresh PBS three times. Following trypsinisation with 0.05% Trypsin/EDTA for 5

minutes, trypsin was neutralised with equal volume of cell media and the solution centrifuged at 16000 RPM for 7 minutes. The supernatant was aspirated and the pellet resuspended in 100ul of RIPA buffer containing 1x protease-inhibitor-cocktail (Complete, Roche, UK) and 1x phosphatase inhibitor (Complete, Roche, UK). The pellet was kept on ice for 10 minutes during cell lysis and then centrifuged at 4 degrees for 20 minutes at 4000RPM. The supernatant was collected, aliquoted and stored at -80 degºC until analysis.

2.2.3.2 Cell protein quantification

The amount of total protein was quantified using the BCA assay. A standard protein curve was obtained following measurement of optical density produced using different concentrations of Bovine Serum Albumin, which were then plotted against each concentration. The optical density was measured at 595nm. The formula for the line of best fit was used to calculate unknown concentrations. A new standard curve was calculated for each assay.

2.2.3.3 SDS Gel Electrophoresis

Cell pellets or cultured media supernatant from cells grown for 48 hr in the absence of serum and supplemented with 50 µg/ml ascorbic acid were used for western blot assays. Loading buffer (6x) containing β-mercaptomethanol was added to each lysate sample and heated at 90 degrees for 5 minutes prior to SDS-gel electrophoresis. A 4-15% Mini-Protean® TGX™ gradient gel (Bio-rad laboratories Ltd, Hemel Hempstead, Hertfordshire) was used. Fibroblast extracts from cultured cell samples were each loaded with a total protein of 50-70 ug of protein and 10ul of Amersham ™ Rainbow™ Full Range molecular weight marker (GE Healthcare UK Limited, Amersham Place, Buckinghampshire,UK) were used. The gel was run at 250V for 90

minutes in 1x Tris/Glycine/SDS Buffer (Bio-rad laboratories) and protein was electrotransferred to a nitrocellulose membrane using a Biorad® TransBlot Turbo at 25 V and 2.5 A for 12 minutes. The membrane was stained with Ponceau Red solution (Sigma-Aldrich) for 5 minutes to check transfer of protein and then washed and incubated in 5% skimmed milk in TTBS at room temperature for one hour followed by overnight incubation with the primary antibody at 4 degrees. The loading control marker used was either βactin or vinculin (See Appendix). The membrane was washed three times for 5 minutes in 1 x Tween20/Tris buffered saline (TTBS) before incubation with a secondary antibody conjugated with horseradish peroxidise (HRP), for one hour at room temperature.

Table 2.2 Antibodies used for protein immunoblotting

Primary Antibody	**Secondary Antibody**
Rabbit polyclonal collagen type VII (antibody targets NC1 domain); kindly sent from Prof Chen, CA; 1:5000	HRP Conjugated swine anti- rabbit (Dako); 1:2000
Mouse monoclonal vinculin (Sigma-Aldrich); 1:250 000	HRP Conjugated goat anti-mouse (Dako); 1:2000

2.2.3.4 Immunodetection

The membrane was washed in 1 x TTBS before the addition of enhanced chemiluminescence (ECL) and Western blotting detection reagent (GE Healthcare, UK). The latter is a substrate of horseradish peroxidase and a positive reaction leads to the production of a luminescent substance which is then visualised by exposure of

the membrane to high performance chemiluminescence film (GE Healthcare) in a dark room. Films were exposed for 1 minute and 10 minutes.

2.2.3.5 Quantification of Western blot data

Image J Software was used to quantify the proteins in each band by performing a densitometry analysis of the TIFF or JPEG image (Schneider et al., 2012). Once the density of each peak was calculated, this was expressed as a percentage of the reference standard, in most cases the standard being a normal control cell type. This was therefore expressed as a percentage value.

2.2.4 Fluorescence Activated Cell Sorting (FACS) analysis of fibroblasts

Cultured fibroblasts were trypsinized using Trypsin 0.05%/EDTA and centrifuged at 500g for 5 minutes at room temperature. The cell pellet was resuspended in media to ensure a concentration of 0.5 x 10 6 cells per 1ml and then centrifuged again. The pellet was then washed with 2ml of FACS buffer (2% FBS in PBS) at 350g for 5 minutes. The supernatant was carefully aspirated and cells resuspended in residual fluid. 100ul of ice cold 4% PFA was added to the cell pellet suspension and incubated for 15 minutes at room temperature in the dark. The cells were then washed with 2.5 ml of FACS buffer and centrifuged for 5 minutes at 350g. The supernatant was aspirated carefully. The cells were then incubated in an antibody dilution buffer (1% BSA, 0.3% Triton X 100 in PBS) containing the primary antibody or IgG3 isotype. Cells were incubated for 30-40 minutes in the dark at room temperature. A sample of unstained cells was prepared for each cell type analysis and incubated with antibody dilution buffer only. Following incubation, the cells were washed with 2mls of FACS buffer, centrifuged and re-suspended in appropriate secondary antibody diluted in antibody dilution buffer and incubated for 30-40 minutes in the dark. Unstained cell

samples were again incubated in dilution buffer alone. Following 2 further washes with FACS buffer, the cells were re-suspended in 350-500µl of FACS buffer and analysed. Acquisition and analysis were performed using an CyAn™ ADP cell analyser (Beckman Coulter Ltd, Oakley Court, High Wycombe, UK) and data analysed with Summit software (Beckman Coulter).

Table 2.3 Antibodies for FACS analysis

Primary Antibody	Secondary Antibody
Mouse monoclonal Antibody type VII collagen LH7.2 (Santa Cruz, UK); 1:25	Goat anti mouse conjugated to AlexaFluor 488 (Invitrogen); 1:200

2.2.5 Viral vector production

Viral vector production took place at the Institute of Child Health (by the GMP trained team). Virions were prepared by transient transfection of validated 293T cells in five-layer cell factories. The culture medium in which particles are harvested was compatible for fibroblast transduction (DMEM and approved source 10% FCS). The crude vector batch was concentrated, pooled and filtrated through 0.22um filters before aseptic filling into cryovials. The vector batch was stored at < -80ºC.

2.2.6 Real-time PCR for assessment of copy number

Total DNA was harvested from cells grown to 70% confluence (Qia-Amp DNA Mini kit, Qiagen, Venlo, Limburg, Netherlands). Integrated vector copy number was assessed in transduced cells after multiple passages by quantitative PCR using primers and probe designed to target the Psi post-regulatory element region of the vector in comparison to signal generated for the housekeeping gene albumin. Integrated copy number was calculated with the aid of standard curves generated using plasmids

encoding the vector and complementary DNA for human albumin. The set of standards comprised plasmid vector containing both the HIV Psi and human albumin sequences (Stock tube 1 is 2.784 x 1011 copies/µl, Genethon, France) and this was serially diluted from 107 copies/µl (108 copy standard) down to 101 copies/µl.

Incorporating a second PCR using primer and probe sequences based on an internal control human albumin, together with appropriate plasmid DNA standards allows the amount of genomic DNA (and thus the number of cells) to be quantified.

Following DNA extraction from cultured cells, the DNA was eluted in DNA/Nuclease free water and the DNA concentration calculated by adding a drop of DNA to the Nanodrop spectrophotometer. For the qPCR reaction, each DNA sample was then adjusted to a concentration of 10 ng/µl for the HIV lentivirus psi qPCR using DNAase/RNAase free water. The PCR reaction comprised the components: 0.25 µl HIV Psi reverse primer (5' TCC CCC GCT TAA TAC TGA CG 3'), 0.25 ul HIV Psi forward primer (5' CAG GAC TCG GCT TGC TGA AG 3'), 0.25 ul Albumin reverse primer, 0.25 ul Albumin forward primer, 0.25ul HIV Psi probe (5' FAM-CGC ACG GCA AGA GGC GAG G TAMRA-3', Reporter is FAM, the Quencher is TAMRA), 0.25ul Albumin probe (5' VIC-CCT GTC ATG CCC ACA CAA ATC TCT CC-TAMRA 3', Reporter is VIC, the Quencher is TAMRA), 12.5ul Platinum Quantitative PCR Supermix-UDG W/ROX (Invitrogen, UK), 1 µl RNAase free H_2O (Qiagen, Crawley, UK) and 10 µl genomic DNA. All PCR reactions were carried out in triplicate, in 96-well plates (ABgene) covered by optical adhesive lids and samples were amplified by the ABI PRISM 7000 sequence detection system and computer ABI Prism software (Applied Biosystems). The reaction was run for 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 seconds and 60°C for 1 min

on a ABI Prism 7000 sequence detection system (Applied Biosystems). Integrated copy number was calculated with the aid of standard curves generated using serially diluted plasmid encoding both viral packaging Psi and endogenous housekeeping Albumin sequences.

Chapter 3

The development of a phase 1 study of *ex vivo* gene therapy using autologous gene corrected keratinocytes and fibroblasts to generate a skin equivalent graft in RDEB patients (GENEGRAFT)

3.1 Introduction

3.1.1 *Ex vivo* gene therapy for RDEB

Gene therapy for inherited skin diseases can be attempted via *in vivo* or *ex vivo* techniques. *In vivo* approaches directly administer the target gene/DNA plasmid or gene containing vector into the skin. Advantages of this approach are the avoidance of surgery and the lack of cell manipulation and prolonged *in vitro* cell culture. Although this method permits the treatment of large areas of skin, the main limitations have been concerns over the bio-safety and bio-distribution of directly administered viral vectors directly into skin and any potential non-target tissue effects (including reproductive organs), as well as possible immunogenicity.

Ex vivo gene therapy circumvents many of these issues. The first step requires an initial skin sampling via biopsy followed by isolation of target cells (keratinocytes and/or fibroblasts) using well-established tissue culture techniques (Rheinwald and Green, 1975). The next stage involves transduction using an appropriate vector containing the target transgene. Genetically cultured autologous cells are then further expanded in culture and transplanted or grafted back onto the patient. Pre-clinical studies using these techniques in models of RDEB are discussed and summarised in Chapter 1. At the time of starting this thesis, there had been no successful cases of *ex vivo* genetically

corrected cells or grafts attempted for patients with RDEB. The broader aims of this work are to develop an autologous skin equivalent graft corrected with a validated retroviral vector carrying the *COL7A1* transgene (SIN RV COL7a1 vector EF1 α). The specific aims within this work include the selection of the optimal patients for grafting.

3.1.2 Project aims

The aim of this study was to design and implement a phase I/II *ex vivo* gene therapy clinical trial for RDEB using skin equivalent grafts genetically corrected with a *COL7A1*-encoding SIN retroviral vector (GENEGRAFT). The application for funding was made in 2009 and was awarded an FP7 grant, based on the collaboration of multiple academic sites within the European Union. The objectives of the GENEGRAFT project are to develop an *ex vivo* gene therapy approach to deliver a permanent treatment locally in RDEB skin. Titeux *et al* (2010) first developed the pre-clinical model and used an SIN retroviral vector with *COL7A1* cDNA under the control of a human EF1α promoter to transduce both primary RDEB keratinocyte and fibroblast cell populations (Titeux et al., 2010). This pre-clinical model was able to demonstrate long term stable *in vivo* engraftment with restored levels of C7 and functioning AFs at the DEJ.

The aims of the GENEGRAFT project (phase 1 trial) were to translate this pre-clinical model into a phase I clinical trial, and treat up to 3 patients selected from centres in both France (IMAGINE institute Paris, Necker Hospital for Children) and the United Kingdom (Guys and St Thomas' NHS Foundation Trust). This would be termed a CTIMP (Clinical trial of an Investigational Medicinal Product) incorporating the whole process including grafting. In this case, the Investigational Medicinal Product (IMP) is a fibrin based skin equivalent graft composed of genetically modified autologous keratinocytes and fibroblasts. The fibroblasts are embedded in a fibrin gel made from cryoprecipitate of human plasma derived from the recipient, which allows further cultivation of keratinocytes to produce a stratified epithelium. Using methods successfully utilised to treat burn patients (Llames et al., 2004, Navsaria et al., 1995)

the aim is to develop an IMP for patients with RDEB. Genetically modified keratinocytes cultured to form epidermal sheets have been developed for patients with JEB (Mavilio et al., 2006) but the addition of a dermal component has a number of advantages both reproducing the natural microenvironment and introducing two cell types both capable of secreting C7.

Following consultation with the UK National Research and Ethics Service (NRES) directors, the project was divided into two parts. The first part of the study is an observational study with no investigational study intervention, aiming to characterise a cohort of patients with RDEB most suited for future *ex vivo* gene therapy interventions. This would be an observational study rather than a CTIMP and therefore would only require regulatory approval from the NRES committee rather than the MHRA. This was done to ensure that the selected patients would be suitable for entry immediately once the CTIMP gained regulatory approval within the European Union.

3.1.3 Workpackages

Being a European wide project, this phase I study was subdivided into work-packages with designated deliverables and responsibilities for each beneficiary. The main roles detailed in this thesis are related to patient selection and recruitment, protocol design, protocol implementation and regulatory applications.

3.2 Methods

3.2.1 Study design

This multicentre prospective study aimed to characterise up to 30 patients with RDEB across all clinical sites in terms of immune response to type VII collagen, epidermal cell proliferative capacity and clinical and biochemical criteria most suited for a study

for *ex vivo* gene correction. The study in France initially assessed those aged 7-65 years whereas the UK study only assessed adults over the age of 18 at study recruitment. In order to initiate the pre-selection process for the GENEGRAFT study, the project was divided into two parts, the primary observational study only examining the clinical and molecular features necessary for patient selection. The secondary study would then involve the delivery of the genetically corrected skin equivalent graft to the selected patients. The UK National Research and Ethics Service (NRES) agreed that a primary "pre-selection" study would be necessary, critically ensuring that all patients consented for pre-selection would not be obliged to take part in the second part. This pre-selection study was entitled EBGen, "Study of immune tolerance and wound healing in patients with recessive dystrophic epidermolysis bullosa". This study involved assessment of clinical features including disease severity and extent of chronic wounds. This was documented using an objective severity score (Birmingham Epidermolysis Bullosa Severity Score, BEBSS) as well as medical photographs. The BEBSS is a validated scoring system takes into account the percentage of cutaneous disease involvement, mucosal and nail involvement, hand scarring and deformity, number of squamous cell carcinomas, EB related alopecia and nutritional compromise (Moss et al., 2009). All patients had an assessment of immune response to C7, utilising ELISA, ELISPOT, HLA Genotyping and Indirect IF. Only a select few would then have a further skin biopsy in order to assess keratinocyte proliferative capacity. The limitation of only up to 2 patients for this assessment was due to cost and time required for each sample process. The design of validated documents for the ethics submission included a clinical protocol for the study, Patient Information leaflet (PIL), Consent form and GP notification letter (See Appendix).

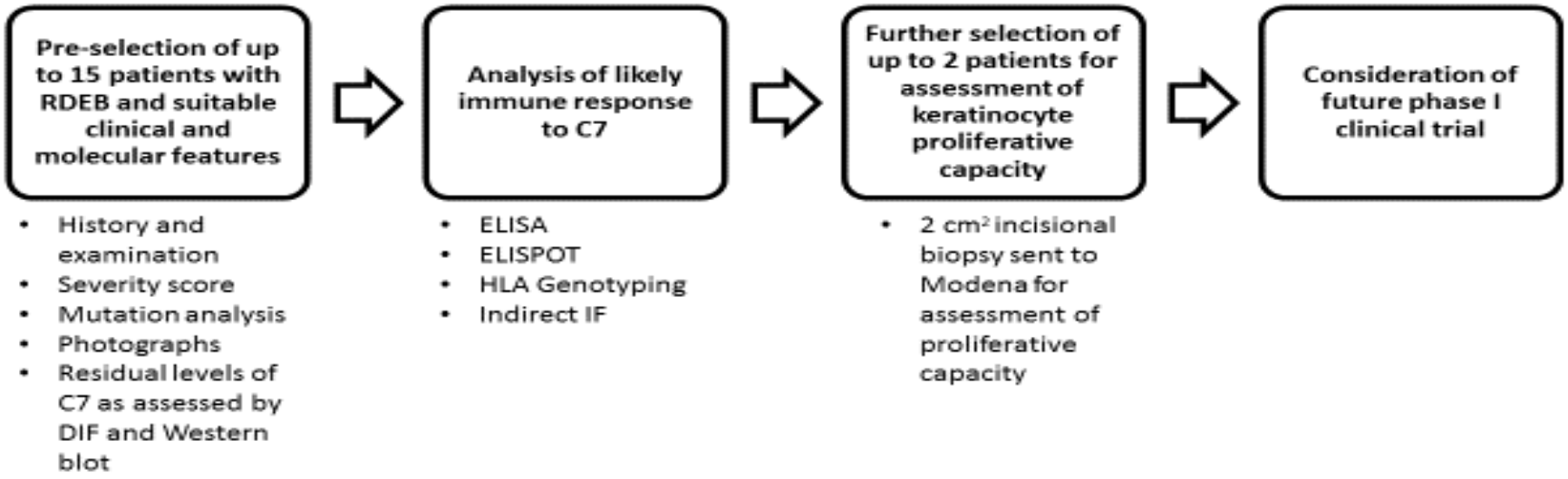


Figure 3-1 Diagram showing EBGen study flowchart and study method

3.2.2 Human subjects

The study EBGen (Study of Immune Tolerance and Capacity for Wound Healing for RDEB, Ref: LO/12/1472)_was approved by London Brent Ethics Committee in October 2012. Subsequently the application to Guy's and St Thomas' Research and Development (R+D) department was submitted via the National Institute for Health Research Coordinated System for gaining NHS Permission (NIHR CSP) which is a streamlined system for gaining R+D permission in the NHS in England for studies that fall under the NIHR Clinical Research Network Portfolio. R +D approval was confirmed on 18th July 2013. For the diagnostic assessment and characterisation of patients suitable for entry into this study, previous research ethics approval by Guy's Research Ethics Committee, Guy's and St Thomas' NHS Trust was used (Characterisation of molecular and structural skin abnormalities in inherited skin disorders, Ref: 07/H0802/104).

3.2.3 Substantial Amendments

There have been three substantial amendments during the study period, as shown in Table 3.1

Table 3.1 Details of EBGen study substantial amendments

Amendment no	Date	Justification
1.0	Jan 2013	The EBGen study was reviewed by the French Ethics Committee (CPP) in Paris on the 13th November 2012. The ethics committee were keen to avoid any mention of future potential gene therapy in relation to this study. This was in order to ensure patient expectations are related solely to the EBGen study. This required modifications to protocol, patient information leaflet and study title.
2.0	June 2013	1) Change of Chief Investigator (CI) to Prof John McGrath. This was due to advice from the CSP group that the CI must be UK based. As such, Prof Hovnanian will be the main EU coordinating investigator rather than the UK CI. 2) Change in laboratory processing the skin biopsy samples for proliferative capacity assessment. This will now be performed in an approved GMP grade laboratory in Modena, Italy, led by Prof Michele De Luca.
3.0	July 2014	Change of inclusion and exclusion criteria to allow inclusion of patients with severe phenotype expressing absent levels of C7 on DIF, providing that C7 can be detected by Western Blot.

3.2.4 Development of pre-selection study protocol (EBGen)

The pre-selection study was designed to screen RDEB patients and select the "ideal candidates" for a phase I study of *ex vivo* gene therapy in RDEB. The definition of the "ideal candidate" has evolved over time and the challenges to determine the most suitable candidate for this approach are discussed in detail. The initial aim was to graft unblistered sites on a patient with moderately severe RDEB (RDEB, generalised-intermediate). Disease severity, co-morbidities, social and family supports are all important factors in determining the likelihood of tolerating a graft procedure. One of the main theoretical risks of the therapy is the development of a systemic immune response to the transgene or new C7 produced. Therefore, individuals were assessed using ELISA and ELISPOT to C7. The main criteria for initial assessment are shown below. Patients deemed suitable according to these set criteria would then undergo a further two skin biopsies for assessment of keratinocyte proliferative capacity

I. History and examination in order to ascertain disease severity, inclusion and exclusion criteria and assess for possible graft sites

II. BEBSS score (Birmingham Epidermolysis Bullosa Severity Score) (See Appendix)

III. Immune tolerance to C7 wild type protein using ELISA and ELISPOT assays

IV. HLA Genotyping to determine the likelihood of immune response to C7 wild type protein

3.2.5 Skin biopsy technique

Using an aseptic technique, local anaesthetic (Xylocaine 2% with adrenaline 1:200,000) was infiltrated into each of the biopsy sites. Full thickness 5mm skin punch biopsies were taken and the wounds closed with prolene sutures or in the cases of

severe RDEB left to heal via secondary intention. Routine wound care advice was given and sutures were removed after 7 days.

3.2.6 Western blot analysis of cultured cells

Protein extraction was performed from cultured fibroblasts and keratinocytes using the methods described previously. SDS Page Gel electrophoresis was then performed using at least 50µg of protein lysate according to previously described methods. Immunodetection was performed using rabbit polyclonal antibodies targeting the NC1 domain of C7 and then membranes were incubated with a secondary antibody conjugated with HRP.

3.3 Results

3.3.1 Patient pre-selection

Patients were screened using the database located at the Robin Eady National Diagnostic Laboratory at St Thomas' hospital. A total of 153 patients were identified and screened from the electronic database with mutation analysis showing heterozygous, compound heterozygous or homozygous mutations in *COL7A1*. From this list of patients, further data was retrieved from medical records and reviewed with the clinical team including the Debra EB specialist nurses. A proforma was devised to filter only patients suitable according to study inclusion and exclusion criteria. This document was used across the EU recruitment sites to collate data. The initial selection criteria targeted a list of patients with moderate disease, classified as RDEB-generalised intermediate. This was based on the assumption (no proven supportive data) that patients with RDEB generalised-severe (RDEB-Gen-Sev) would have a complete absence of C7 and therefore be at much higher risk of developing an adverse

immune response to wild type C7. According to these criteria there were 10 suitable patients for consideration. Clinical and molecular data are shown in Table 3.2.

Study No	Details	Clinical Diagnosis	Mutation Analysis	C7 Immunostaining	Western Blot C7	Comment
EB1	51 (M)	RDEB (Inv)	MS/MS	+++	++	Oral disease and flexures
EB2	39 (F)	RDEB (Gen Int)	GS/GS	N/A	N/A	Mild cutaneous involvement
EB3	69 (F)	RDEB (Gen Int)	PTC/GS	++	++	Coexistent autoimmune disease requiring oral steroids
EB4	28 (F)	RDEB (Gen Int)	MS/MS	++	N/A	Mild cutaneous involvement; social concerns with 2 young children
EB5	60 (F)	RDEB (Gen Int)	FS/GS/GS*	++	++	Oral disease, mild cutaneous involvement
EB6	53 (F)	RDEB (Gen Int)	GS/GS	+++	N/A	Worsening cutaneous disease over time
EB7	40 (F)	RDEB (Inv)	PTC/--	---	--	Severe oral and mucosal involvement, minimal cutaneous disease
EB8	33 (M)	RDEB (Gen Int)	FS/MS	+++	++	Predominantly lower leg erosions
EB9	28 (M)	**RDEB (Inv)**	Del/GS	++	Contaminated cells	Recalcitrant neck wounds, social issues and previous IVDU
EB10	28 (M)	RDEB (Gen Int)	Homozygous FS	+	--	Motivated, minimal chronic wounds

Table 3.2. Patients with RDEB (Gen Inv) selected showing C7 immunostaining and Western blot results

MS = missense, GS = Glycine substitution, PTC = Premature termination codon, FS = Frameshift, M = male, F = female, N/A = not assessed, IVDU = Intravenous drug user, Del = deletion, Gen Int = Generalised Intermediate, Inv = Inversa.

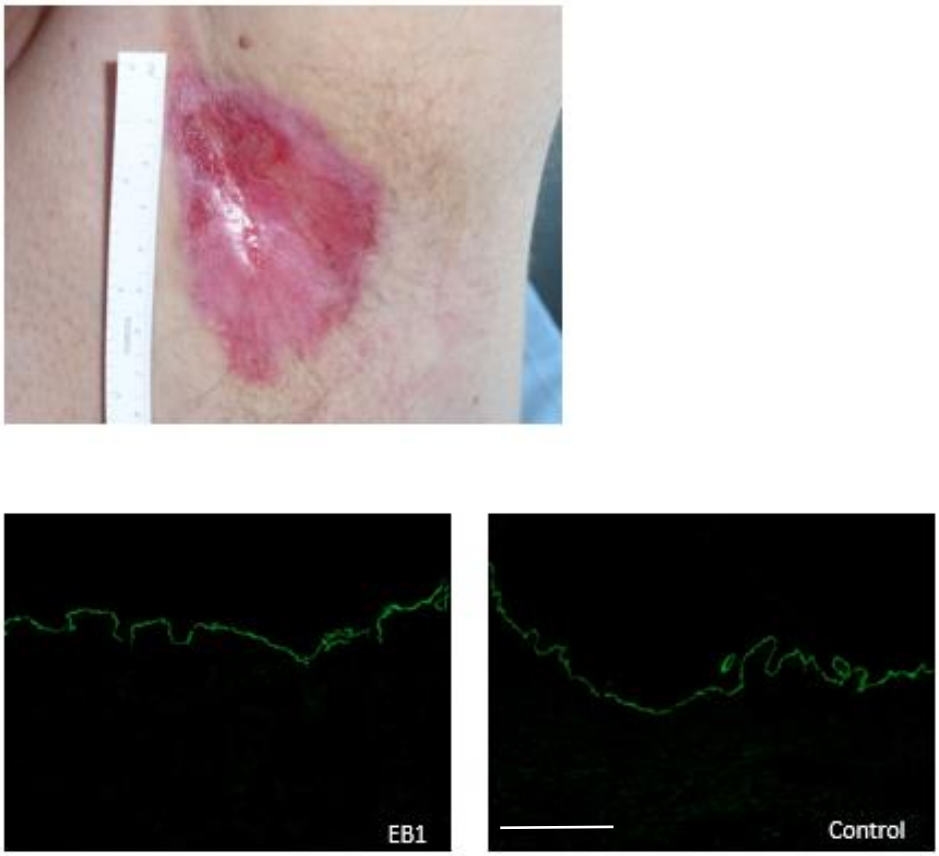


Figure 3-2 The clinical phenotype of a patient with RDEB (Inversa) and correlated with immunofluorescent labelling of skin with anti-type VII collagen antibody, LH 7.2.

The patient labelled EB1 showing a longstanding non-healing erosion on the left axilla. The immunofluorescent labelling with anti-type VII collagen antibody in EB1 skin sections compared with controls, showing linear labelling with subtle slight discontinuous staining in some focal places. Due to the mild phenotype and almost normal levels of C7 present on DIF this patient was not selected for the EBGen study.

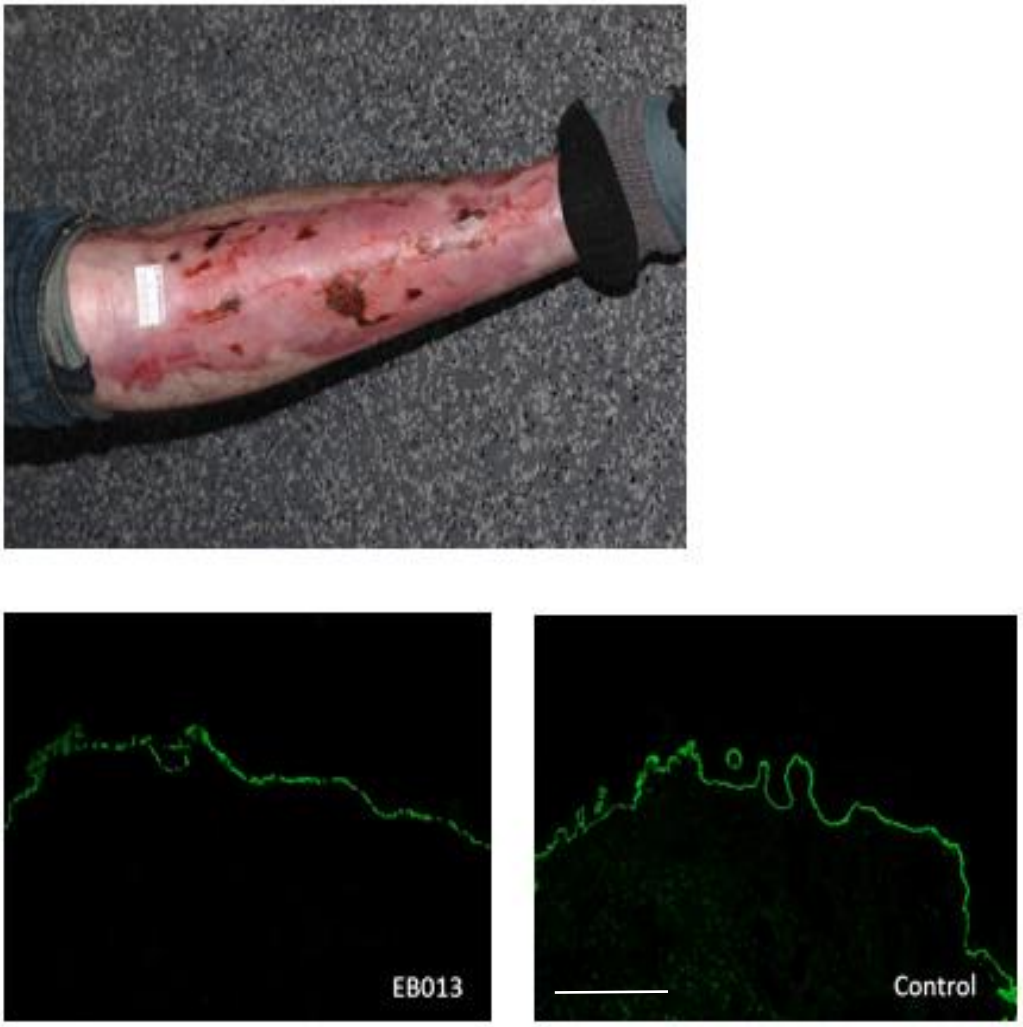


Figure 3-3 The clinical phenotype of a patient with RDEB Generalised Intermediate and correlated with immunofluorescent labelling of skin with anti-type VII collagen antibody, LH 7.2.

The patient labelled EB013 showing a large erythematous plaque on the anterior leg with overlying excoriations and erosions. The immunofluorescent labelling with anti-type VII collagen antibody in EB013 skin sections compared with controls, showing linear labelling with subtle slight discontinuous staining in some focal places. The patient had severe disease affecting only the lower legs. Due to the perceived difficulty grafting these areas and the almost normal levels of C7 present on DIF, this patient was not selected for the EBGen study. Scale bar =100µm.

3.3.2 Subject details and molecular diagnoses

Following further consideration and review of this patient cohort, a change to the protocol was made in order to include patients with RDEB (Gen Sev). On reflection, patients with only moderate disease would be far less likely to be willing to undergo a grafting procedure of their "normal skin". Furthermore, the desired increase in levels of C7 following gene correction strategies would be less apparent via DIF and Western Blot in patients with near normal levels of C7. In total nine patients with RDEB (Gen,Intermed) or RDEB (Gen Sev) were suitable according to amended inclusion and exclusion criteria (see Section 3.3.3). All patients presented with generalised blistering at birth or shortly afterwards. The most frequently affected areas were the back, neck, upper legs, hands and feet. Only 2 had developed mitten deformities and 6/9 had required previous hand surgery. Swallowing difficulty or dysphagia was reported in 5/9. For 6/9 patients oesophageal strictures were evident and 4/9 required repeated oesophageal dilatations. One patient had renal failure requiring regular renal team input (glomerulonephritis). At study entry none of the patients had a squamous cell carcinoma. All recruited patients had anaemic periods requiring intravenous iron therapy, and Patient BH-01 required intermittent blood transfusions. Of note, 5/9 of the cohort had received previous intradermal allogeneic fibroblast therapy as part of the phase II study of intradermal allogeneic fibroblast therapy for chronic wounds in adults with RDEB (ISRCTN6775729)(Petrof et al., 2013). The study had completed in 2013 and most had shown a beneficial impact on wound healing although only transiently (up to 28 days only), and therefore would assume no impact on future inclusion in cell or gene therapy clinical trials.

3.3.3 Study inclusion and exclusion criteria

Inclusion criteria

1. Confirmed molecular diagnosis of recessive dystrophic epidermolysis bullosa, established for both alleles;
2. Moderate to severe RDEB;
3. Presence of the NC1 domain type VII collagen on skin biopsy and/or western-blot analysis detected with a set of specific antibodies;
4. Presence of blistered or erosive areas, including chronic wounds;
5. No recent hospitalization related to EB complications;
6. Subjects and their parents when applicable should be able and willing to return for follow up;
7. Patients should be able and willing to give signed informed consent.
8. Ability to undergo local anaesthesia.

Exclusion criteria

1. Severe disease indicated by
    a. Absence of detectable type VII collagen expression on skin biopsy and Western blot analysis from cultured cells;
2. Underlying conditions, diseases or active infections likely to increase the risk of complications including

a. Active infectious diseases, including systemic infections and known positive HIV serology (Kaposi's sarcoma), hepatitis B and C;
b. History of current psychological or psychiatric disease;
c. Absence of an adequate familial and social support;
d. History of current or previous organ diabetes mellitus;
e. Non corrected severe anaemia (Haemoglobin level: < 8 g/ml);
f. Non corrected iron deficiency;
g. History of significant allergy to anaesthetic procedure
h. Patient currently receiving anticoagulant or anti-platelet treatment;
i. Positive pregnancy urinary test or lactating women

Figure 3-4 Clinical features and immunofluorescence results for BH-01

Patient BH-01 had chronic wounds affecting the axillae, neck and lower legs. The immunofluorescent labelling with anti-type VII collagen antibody in BH-01 skin sections compared with controls, showing occasional areas of punctate staining and fragmentation and interruption of staining in a few areas. Scale bar = 100 µm.

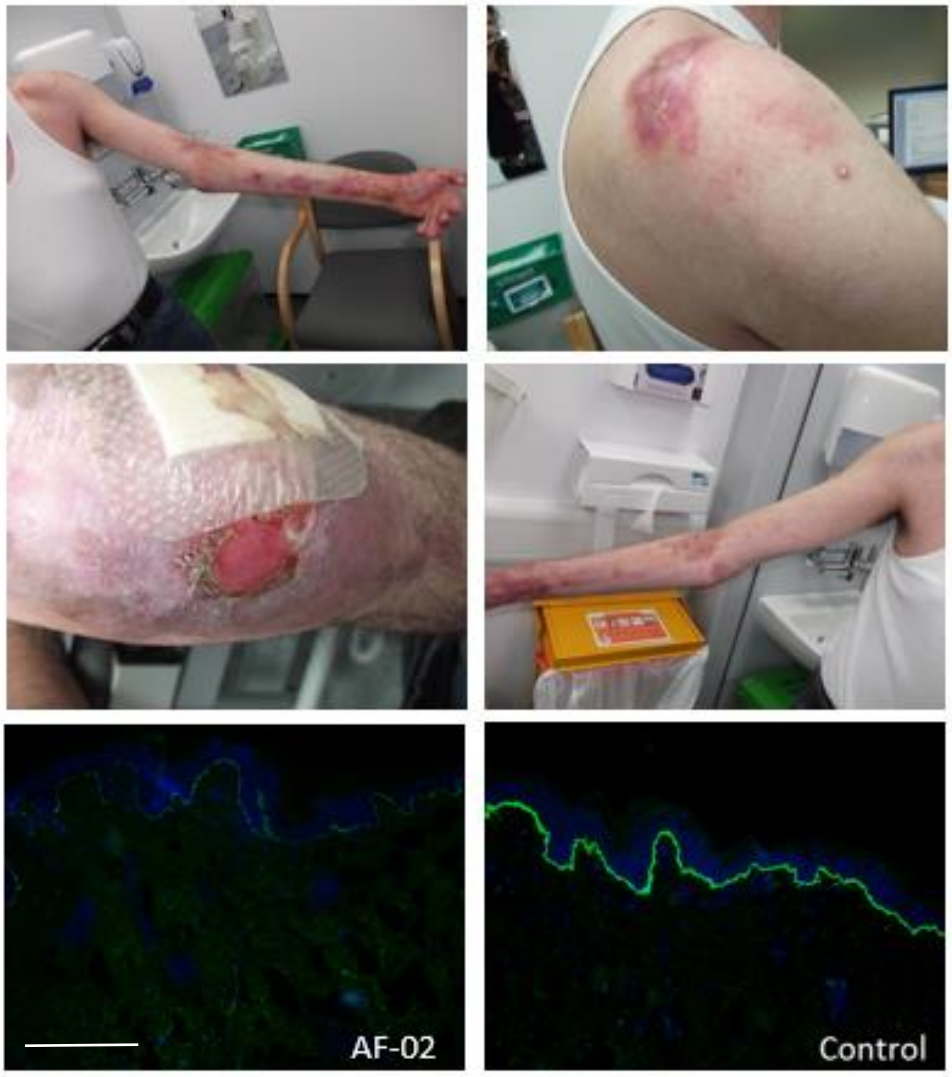


Figure 3-5 Clinical features and immunofluorescence results for AF-02.

Patient AF-02 has generalised fragile skin and some superficial erosions but no chronic wounds. Immunofluorescent labelling with anti C7 antibody shows reduced staining intensity and focal interruptions. Some DAPI nuclear staining is also shown to highlight the epidermis. The extent of C7 reduction is not mirrored by the relatively mild clinical phenotype. Scale bar = 100µm.

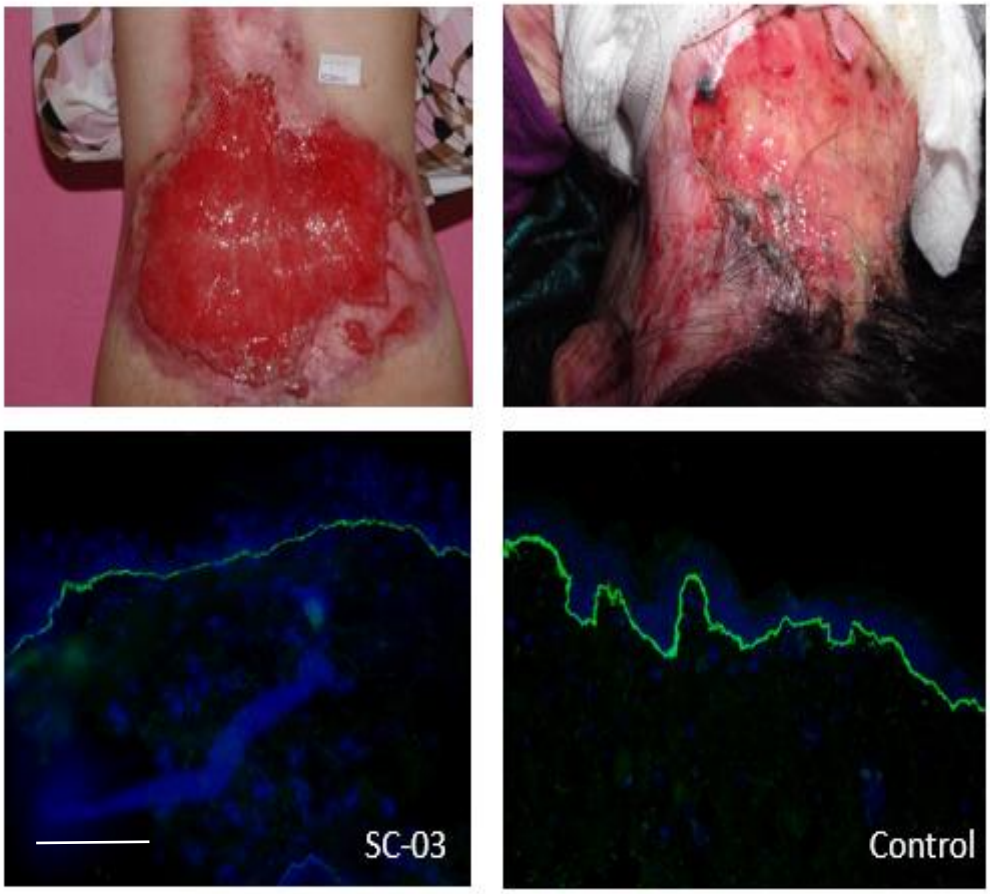


Figure 3-6 Clinical features and immunofluorescence results for SC-03

Patient SC-03 has RDEB (sev-gen) with extensive chronic wounds affecting her back and posteriod neck. . Immunofluorescent labelling with anti C7 antibody shows reduced staining intensity. Scale bar 100 μm.

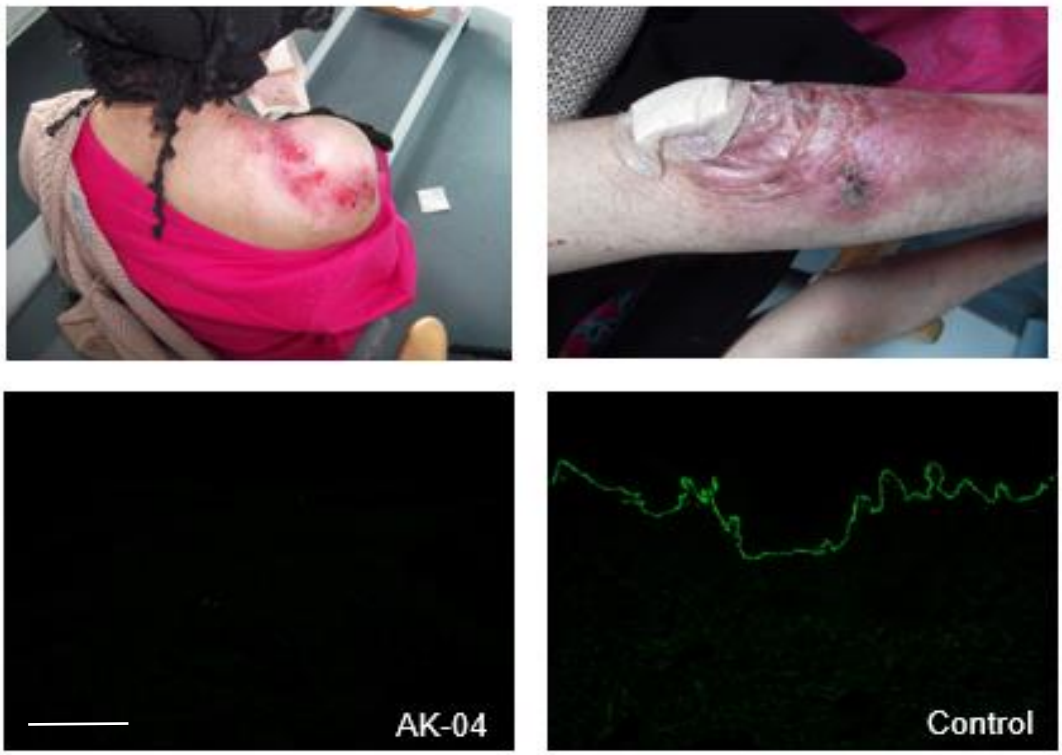


Figure 3-7 Clinical features and immunofluorescence results for AK-04

Patient AK-04 has RDEB (sev-gen) with skin fragility and erosions evident particulary over the shoulders and lower legs. Immunofluorescent labelling with anti C7 antibody shows complete absence of C7. Scale bar = 100µm.

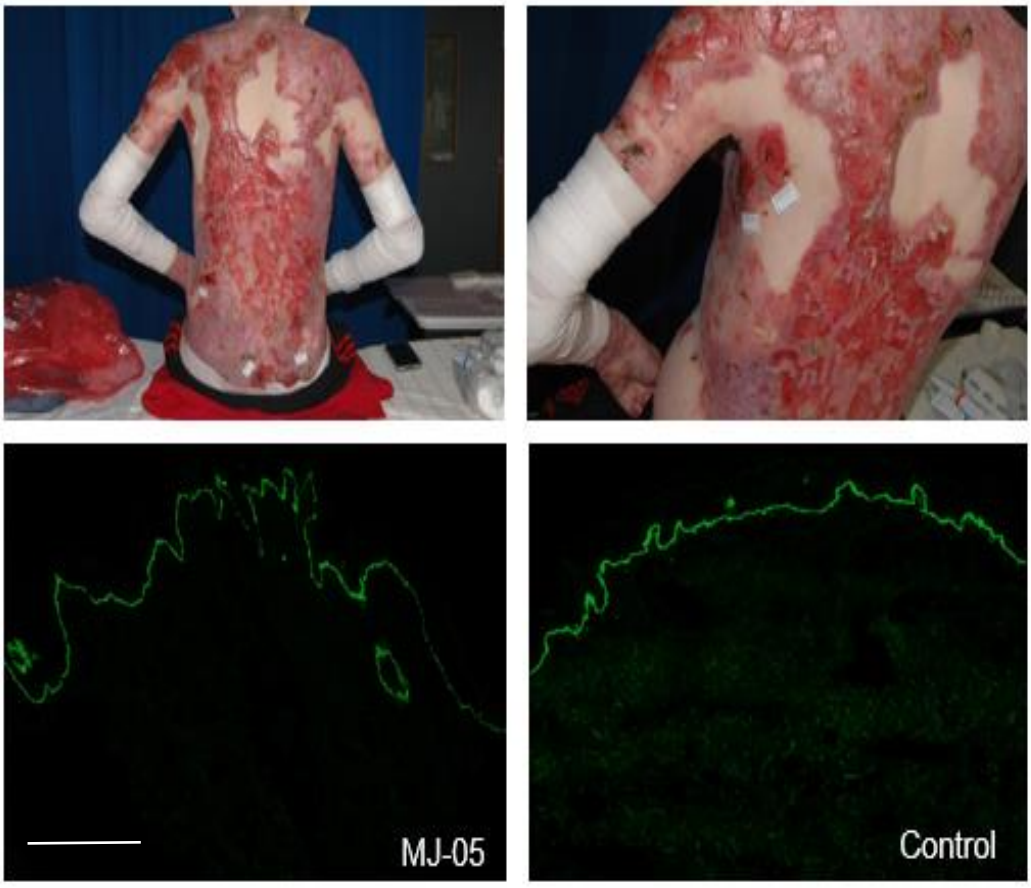


Figure 3-8 Clinical features and immunofluorescence results for MJ-05

Patient MJ-05 with RDEB (Sev-Gen) showing extensive chronic wounds affecting his entire back and both shoulders. Immunofluorescent labelling with anti C7 antibody shows some linear labelling below a separated epidermis. Although the intensity is reduced, the labelling is still reasonably bright. Scale bar = 100µm.

Figure 3-9 Clinical features and immunofluorescence results for HS-06

Patient HS-06 has RDEB (sev-gen) with extensive chronic wounds affecting her back. Immunofluorescent labelling with anti C7 antibody shows reduced staining intensity and occasional interruptions to the linear staining pattern. Some DAPI nuclear staining is also shown to highlight the epidermis. Scale bar = 100µm.

Table 3.3 Clinical characteristics of 9 UK patients recruited for EBGen study

Patient No.	Age (Sex)	Clinical diagnosis	Dysphagia	Hand deformity	Worst affected areas	% area chronic wounds/erosions	Iron Deficiency Anaemia	Previous allogeneic fibroblast therapy	BEBS Score (0-100)	Significant comorbidities
BH-01	52 F	RDEB (Gen, Intermed)	Y (x 5 OD)	Contractures and proximal webbing	Back,Left shoulder and axilla, right knee	30	Y	N	33	Recurrent severe iron deficiency anaemia
AF-02	28 M	RDEB (Gen, Intermed)	N	Mitten deformity Finger release 2003	Elbows, hands, knees, dorsum feet	10	N	N	24	Nil
SC-03	26 F	RDEB (Gen, Intermed)	Y (Stricture 2012)	Right hand surgery	Posterior neck and lower back	10	Y	Y	23	Anxiety and depression
AK-04	23 F	RDEB (Gen, Intermed)	N	Hand surgery	Knees, elbows and right shoulder	35	Y	N	27	Nil
MJ-05	48 M	RDEB (Gen Intermed)	N	Hand surgery	Back, legs and forearms	60	Y	Y	42	Hypothyroidism, contractures both knees
HS-06	23 F	RDEB (Gen Sev)	Y (x 4 OD)	Obvious contractures and proximal webbing	Back and posterior neck	40	Y	Y	47.5	Corneal transplant 2007, Gastrostomy 1996
CK-07	29 M	RDEB (Gen Sev)	Y (x 5 OD)	Finger release x 3	Back, forearms and posterior thigh	30	Y	Y	32	Acute Renal Failure Severe left ventricular Impairment, Previous thyroid dysfunction
WB-08	37 M	RDEB (Inversa)	N	N	Arms and back	45	N	N	25	Depression
JD-09	22 M	RDEB (Gen Sev)	Y (>10 OD)	Hand surgery x 3	Hands, arms, legs and back	70	Y	Y	50	Gastrostomy, wheelchair bound

Abbreviations: Gen Intermed = Generalised Intermediate, Gen Sev = Generalised Severe, M= male, F= female, OD = oesophageal dilatation, RDEB = recessive dystrophic epidermolysis bullosa, Y = Yes, N = No.

Table 3.4 Mutation analysis for recruited RDEB patients (performed by Robin Eady National Diagnostic EB lab)

	Mutation 1					Mutation 2				
Patient No.	**Nucleotide**	**Amino acid**	**Exon**	**Consequence**	**Domain**	**Nucleotide**	**Amino acid**	**Exon**	**Consequence**	**Domain**
BH-01	c.5720-5721GA>AT	p.Gly1907Asp	68	Glycine Substitution	Triple helix	c.5552G>A	p.Gly1851Glu	65	Glycine Substitution	Triple helix
AF-02	c.8506insC	p.Val2836fsX12	115	PTC	NC2	c.8506insC	p.Val2836fsX12	115	PTC	NC2
SC-03	IVS 13-1G> C			Splice site	NC1	IVS 13-1G>C			Splice site	NC1
AK-04	c.5660_5661delGGinsATA	p.Gly1887fsX27	67	PTC	Triple helix	c.5660_5661delGGinsATA	p.Gly1887fsX27	67	PTC	Triple helix
MJ-05	c.1732C>T	p.Arg578X	13	PTC	NC1	IVS20+2T>C			Splice site	
HS-06	c.7139G>A	p.Gly2380Glu	93	Missense	Triple helix	IVS115-1G>A			Splice site	NC2
CK-07	c.425A>G	p.Lys142Arg	3	Splice site	NC1	c.425 A>G	p.Lys142Arg	3	Splice site	NC1
WM-08	c.3480delC	p.Gly1280ValfsX44	31	PTC	Triple helix	c.8780G>A	p.Arg2927His	117	Missense	NC2
JD-09	c.425A>G	p.Lys142Arg	3	Splice site	NC1	c.565C>T	p.Gln189X	5	PTC	NC1

Abbreviations: PTC = premature termination codon, NC1 = non-collagenous domain 1, NC2= non-collagenous domain 2

3.3.4 Chronic wound microbiological assessment

RDEB patients have chronic wounds that are commonly colonised. Graft infection is a significant anticipated risk in this patient cohort. Of all the study patients, n= 6/9 have current or previously documented history of bacterial colonisation of chronic wounds with a range of organisms most commonly Staphylococcus aureus sp, and Streptococcus sp. One patient (BH-01) had a previously treated MRSA positive skin infection and unusually one of the patient group developed a cutaneous infection of Diphtheria isolated from a chronic wound over the back. This was discussed with the microbiology team who advised this may have been an unusual skin contaminant or commensal but was treated with two weeks of oral macrolide antibiotics and full screening was subsequently undertaken for the EB patient cohort. Wound colonisation would have a definite impact on healing and increases the risk of graft failure. The results of wound swabs taken from chronic wounds within this patient cohort are summarised in Table 3.5.

Subject number	Microbes isolated	Site	Perceived risk for grafting
BH-01	*MRSA* (treated 2008)	Nose	High
	Staphylococcus aureus (mild)	Back/Chest	--
	Streptococcus dysgalactiae (Group C/G)	Knee	High
AF-02	*Staphylococcus aureus*	Ear	--
SC-03	*Staphylococcus aureus*	Neck	--
	Pseudomonas aeruginosa	Neck, back	--
AK-04	*Staphylococcus aureus*	Back	--
MJ-05	*Heavy growth of Acinetobacter*	Right thigh	--
HS-06	*Staphyloccocus aureus*	Back	--
	Streptococcus Group C	Back	High
	Diphtheria	Back	High
CK-07	*Pseudomonas aeruginosa*	Thigh	--
JD-08	*Streptococcus dysgalactiae, Pseudomonas aeruginosa, Staphylococcus aureus*	Arms	High
WM-09	*Nil*	--	---

Table 3.5 Microbiology assessments of wound swabs taken from recruited patients and predicted risk of wound infection following grafting

Wound swab results were reviewed with a consultant microbiologist at GSTT and given a subjective opinion of those organisms most likely to lead to graft failure (labelled "High")

3.3.5 Results of type VII collagen expression analysis

The presence of C7 was assessed using direct immunofluorescence on skin biopsy using a monoclonal antibody to the NC1 domain of C7 (Mouse monoclonal Antibody type VII collagen LH7.2 (Chemicon Europe Hampshire, UK); 1:100). Each sample was compared with a wild type control during each run, and a negative control used to assess for non-specific binding of the secondary antibody. 3/9 patients had almost completely absent levels of C7 on DIF. Patient subjects' skin biopsies showed varying levels of staining ranging from minor interruptions in staining at the DEJ to complete absence of C7.

Study No.	C7 immunostaining on Direct Immunoflouresence (DIF)
BH-01	++
AF-02	+
SC-03	+
AK-04	-
MJ-05	++
HS-06	++
CK-07	-
WM-08	++
JD-09	-
WD-10	+++

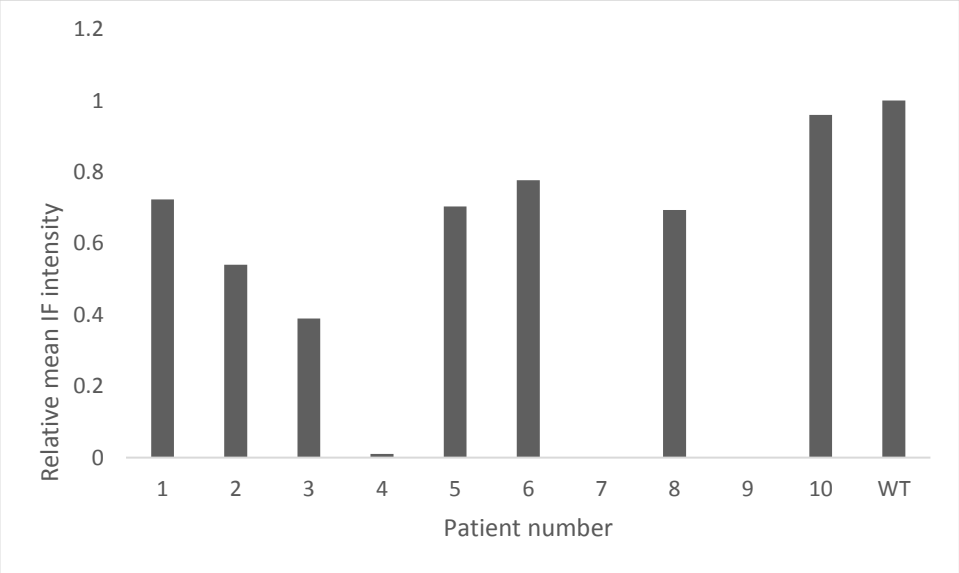


Table 3.6 Immunolabelling for type VII collagen in patient subjects

Subjective measures of intensity are shown in the table when verified with the reporting team at the National Diagnostic EB laboratory (+++) = normal (++) = slightly reduced pattern of staining, (+)= faint staining, (-)= complete absence. Using identical image settings three measurements the relative mean fluorescence intensity of C7 fluorescence signals was calculated using Image J software compared to wild type (WT) normal control DEJ signal.

3.3.6 Western blot detection of C7 protein from cultured cells

The amount of residual levels of C7 protein can be extracted from cultured cells and analysed using Western blot. Western blot analysis would be able to demonstrate any traces of residual non-functioning or aberrant levels of C7. This would be particularly useful in patients that exhibit almost completely absent levels of C7 on DIF. There were challenges in culturing keratinocytes from patients with RDEB, but fibroblast lysates were isolated from each patient. As keratinocytes express far higher levels of C7 protein than fibroblasts, further stimulation of cell cultures were shown to be necessary in order to elicit the C7 band. Ascorbic acid has been shown to increase secretion of C7 from dermal fibroblasts and TGF-β has also been shown to stimulate fibroblasts in culture (Amano et al., 2007). Ascorbic acid was added to cell cultures at least 48 hours before protein extraction in order to increase levels of C7 detectable in fibroblast samples by Western blot. All patients included in this study had varying levels of C7 using Western blot. Due to the difficulties encountered with blots from fibroblast cultures, it was not possible to run all samples in one simultaneous run with identical conditions. Representative results are shown in Figure 3.10.

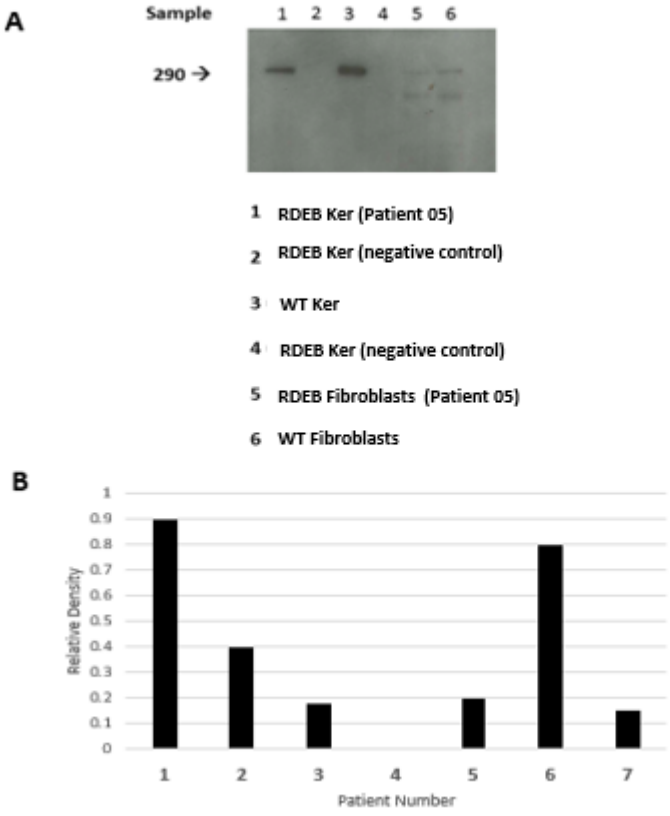


Figure 3-10 Western blot showing bands at the level corresponding to 290 kD from cultured cell lysates

(A)Samples were collected from cultured fibroblasts and keratinocytes. Fibroblast and keratinocyte extracts from cultured cell samples were each loaded with a total protein of 50-70 ug of protein. Immunodetection was performed using rabbit polyclonal antibody directed against the NC1 domain of C7, 1/3000. Samples show comparison of patient keratinocyte and fibroblast lysates (in this case patient 05) with a negative control (EB sample containing absent C7) and wild type positive controls (B) The relative quantities of C7 protein detected using western blot from study patients 1-7 were calculated using Image J software and expressed as a relative proportion.

3.3.7 Immune response to type VII collagen

These results are described in Chapter 4

3.3.8 Selection of patients for assessment of keratinocyte proliferative capacity

The first patient selected for assessment of keratinocyte proliferative capacity was MJ-05. This assessment determines the time taken for generation of an epidermal sheet

and therefore indirectly a measure of the number and function of epidermal stem cells capable of sustained proliferation. This is an essential criterion in terms of patient selection for this study which utilises keratinocytes isolated from skin biopsy in order to generate a long terms skin equivalent graft. In RDEB there is a significant risk of keratinocyte stem cell "exhaustion" due to continuous and recurrent blistering. Those patients considered most suitable for grafting should have initial assessments of keratinocyte proliferative capacity. In this cohort, keratinocyte proliferative capacity may be determined by i) subject's age ii) biopsy sample location iii) sampling conditions iv) transport and culture conditions as well as the effects of the underlying C7 deficiency. Clinical observations have suggested that epidermal stem cell numbers or function may be depleted with increasing age (Barrandon and Green, 1987, Youn et al., 2004), however this has not been borne out in murine models. Charruyer *et al* (2009) have shown that slower wound healing in ageing mice may be due is not due to a decline in stem cell potential but rather the decreasing proliferative output of progenitor cells (Charruyer et al., 2009). Slower wound healing in ageing skin may therefore not represent an absolute reduction of EpSCs (Racila and Bickenbach, 2009, Gago et al., 2009) but rather impaired stem cell mobilisation and ability to respond to recruitment signals. Scientific observations from the centre of regenerative medicine in Modena have suggested that the number of holoclones isolated from skin biopsy samples as assessed via colony forming assays is related to subjects' age (personal communication, Michele De Luca, November 2015). Clonal analysis of keratinocytes in culture also varies according to location in the human cornea (Builles et al., 2008) and the likely determinant of stem cell numbers in human skin would be the amount of hair follicles as well as the thickness of the epidermis. Based on the location of human epidermal stem cells within both the hair follicle bulge and upper isthmus, hair-

bearing sites would be preferred for sampling. However, Mavilio *et al* (2006) observed that only the hairless palmar aspects in their subject yielded sufficient holoclones for generation of an epidermal graft. Therefore, one would assume that sites with thicker epidermis may have higher numbers of epidermal stem cells within the interfollicular region or that the relatively unblistered sites may have preserved numbers of epidermal stem cells. However, this study also demonstrated that donor biopsy site skin cells still retain the characteristics of that anatomical location through serial doublings. Keratin 9 expressed exclusively in keratinocytes isolated from palms and soles was still detected in the transgenic epidermis 6.5 years after grafting (De Rosa et al., 2014). This clearly demonstrates the presence of a few epidermal stem cells able to maintain the characteristics of the donor site despite multiple epidermal turnovers.

The skin biopsy taken from MJ-05 was therefore taken from an unblistered hair-bearing site. This was an incisional biopsy measuring $2cm^2$ taken from the right abdomen. The transport media and containers for transport were received from Modena 24 hours before the biopsy and all measures taken to ensure that the designated containers were maintained between 2-8ºC alongside the temperature loggers. To ensure transport according to GMP standards, all timings of delivery of the containers, removal of skin biopsy and delivery of the sample had to be documented in pre-prepared logs.

Figure 3-11. Photograph of transport materials and packaging required for transport of skin biopsy according to GMP protocol

The biopsy site was shaved to minimise the risk of contamination and then cleaned with Betadine solution followed by 70% ethanol. Excess disinfectant was removed with sterile saline solution and then the local anaesthetic was injected intradermally. Mepivicaine hydrochloride was used as lignocaine hydrochloride has been shown to exert toxic effects on keratinocyte cell cultures (Mountford et al., 1991).

Samples were then analysed in Modena in order to assess stem cell proliferative capacity according to methods described by Mavilio et al (2006). The results are discussed in the next section.

3.3.9 Selection of patients most suited for phase I clinical trial

Following the final review of data described in this chapter there were three patients felt to be most suitable for consideration of entry for a phase I clinical trial of *ex vivo* gene therapy for RDEB. The main criteria for assessment were as follows

1. A strong desire and motivation to take part.
2. Mutations on both alleles of *COL7A1* predicting a severe disease phenotype.
3. Minimal amounts of C7 demonstrated on either WB or IF reducing the likelihood of an immune response to gene therapy product increasing C7 levels.
4. Blistering or wounding in areas best suited for surgical grafting and ease of dressing changes.
5. Absence of anti-C7 antibodies binding to DEJ as evidenced by IIF.

The first patient to be considered was CK-07. This patient has a strong desire for participation and has blistering affecting areas such as the forearms, a relatively straightforward area for grafting. In terms of immune response to C7 this patient had a negative ELISPOT and IIF result, with only a borderline positive ELISA using one of the assays. This patient has a homozygous mutation c.425A>G in exon 3. Gardella *et al*. have previously demonstrated this variant is a pathogenic splice site mutation (-2 position of the splice donor of intron 3) through the creation of three out of frame transcripts each resulting in a PTC (Gardella et al., 1996). Therefore one would expect

complete absence of C7 protein. This was confirmed on immunofluoresence but unusually a small amount of C7 was demonstrated on Western blot from cultured keratinocytes only (not fibroblasts).

The second patient to be considered was HS-06. The key determinant for inclusion has been her strong desire to graft troublesome wounds on her back that have been present for over 10 years. Despite a relatively severe clinical phenotype she has evidence of C7 both on IF and Western blot analysis using antibodies against the NC1 domain. This patient has two heterozygous mutations in *COL7A1*; a glycine substitution c.7139G>A in exon 93 leading to p.Gly2380Glu and an acceptor splice site mutation IVS115-IG>A likely to result in exon skipping or aberrant splicing, which may account for the higher than expected levels of C7 demonstrated. Immune response was favourable as she only had a borderline positive ELISPOT but ELISA and IIF were negative. One of the clinical concerns would be the risk of wound infection at graft site as she had previously been found to have positive Diphtheria swab showing a toxic strain. This has been appropriately treated and wound swabs from multiple sites would need to be taken before undertaking a surgical procedure.

The third patient for consideration was MJ-05. This individual is highly motivated and keen to be involved in a clinical trial. This patient has mutations resulting in a PTC on exon 13 (p.Arg578X) within the NC1 domain and a splice site mutation IVS20+2T>C. The location of chronic wounds again includes the back area, which could be difficult in terms of grafting unless he would be able to sleep on his front during the post-operative period. There was demonstrable C7 on both IF and Western blot from cultured cells and no evidence of an immune response to C7 based on ELISPOT and IIF analysis, although both ELISA assays were positive strongly

suggesting the presence of some anti-C7 antibodies that do not bind to the DEJ. These antibodies may be a consequence of previous therapy with intradermal allogeneic fibroblasts, although there is currently no direct evidence to support this theory.

3.4 Discussion

The aim of this study was to select a suitable cohort of patients for a phase I study of *ex vivo* gene therapy for RDEB. The definition of the "ideal patient" has evolved over time. Initially patients with only mild to moderate disease, classified as having RDEB (Gen Intermed) or RDEB (Inversa) were identified, rather than RDEB (Gen Sev). Patients with moderate severity disease would be likely to have varying amounts of aberrant or non-functioning C7 as opposed to those patients with RDEB (Sev-gen) who typically have minimal or absent levels of C7. Those with moderate disease hypothetically have a reduced risk of developing a significant immune response to an engineered graft containing exogenous C7 (Pendaries et al 2010). Patients with moderate disease severity would also be more likely to have large areas of unblistered skin suitable for grafting. Conversely the tolerability and ethical considerations of performing an invasive procedure such as skin grafting in a moderately affected patient need to be considered. The majority of patients with RDEB (Gen Intermed) or RDEB (Inversa) had a strong preference for therapies targeting their chronic wounds, which were very localised. Approaching these patients with a trial protocol designed to graft an area of normal appearing skin with the associated risks of scarring would be challenging. Demonstrating graft success using an increase of levels of C7 would also be difficult in those secreting higher levels of baseline mutant protein. Based on these points, only patients with multiple chronic wounds and moderately severe/severe disease were selected.

Further consideration critical to patient selection was subject age at time of grafting. From a purely biological perspective selection of a younger patient should ensure a higher likelihood of optimal keratinocyte proliferative capacity and ensure higher number of epidermal stem cells isolated and cultured from skin biopsy. This is based on unpublished experience of cell culture of RDEB patient samples and comparison of keratinocyte clonal capacity between adult and paediatric patients (discussion with Matthias Titeux, July 2014). From an ethical perspective, one should consider that clinical trials should be targeted at the paediatric population primarily in order to prevent the inevitable and devastating scarring leading ultimately to lethal squamous cell carcinomas in adulthood. Clinical trials in adults whom already have mutilating scars and contractures will have a different impact clinically compared with therapies administered to infants. Clinical trials in paediatric populations in the first instance have been performed for cell therapies such including BMT and intravenous MSC therapy. One consideration is that young children would be least likely to tolerate grafting procedures requiring strict wound care and with a high risk of graft failure due to infection. Steps necessary for optimal post-surgical wound care in young children would be challenging to implement, such as dressing care and avoidance of tampering with the dressing and wound site. The main consideration for phase I studies utilising gene therapies is the unknown risks related to the insertional mutagenesis and possible tumorigenesis. The adverse events occurring in the early SCID trials leading to the deaths of two children due to leukaemias directly related to the gene therapy product still clouds the field. Regulatory bodies are understandably cautious and therefore the preference would be for phase I gene therapy studies to be trialled in adults first and offered to children only once shown to be safe.

Graft site is one the critical factors determining the clinical outcome. One of the most challenging considerations has been whether to place the graft onto unblistered patches of skin versus previously wounded or ulcerated areas. An unblistered site provides a clean and even wound bed suitable for grafting. The level of wound cleavage and wound size would then be standardised between patients. However, the risk of creating a new artificial wound in an area that had previously been unaffected may be extremely unappealing from a patient perspective. Counselling regarding the risk of graft failure and the possibility of creating an artificial wound is difficult. The alternative option of grafting chronically wounded areas is fraught with difficulties, predominantly related to microbial colonisation, but certainly more appealing from a patient perspective. The ideal clinical case would be to graft a freshly blistered wound. New blister sites have higher amounts of recruited epithelial progenitors released in response to the hypoxic stimulus. Necrotic keratinocytes are known to secrete the non-histone nuclear protein, high mobility group box 1 (HMGB1) into the RDEB blister roof. Tamai *et al*. have shown HMGB1 to be released by keratinocytes within the first 10 min after blister formation and to readily enter the bloodstream (Tamai et al., 2011b). This group also identified a subpopulation of bone marrow cells containing epithelial progenitors contributing to the restoration and maintenance of the epidermis. These cells are non-haematopoietic MSCs with cell sorting showing that they were c-kit negative, lineage (Lin) negative and PDGFRα- positive. These cells were shown to be directly mobilised by HMGB1 released from freshly wounded skin, the focal recruitment being guided by the higher local tissue concentration of HMGB1 in the damaged skin as compared with the circulation (Iinuma et al., 2015). Higher levels of these epithelial progenitors would likely lead to improved wound healing and likely successful graft take. Fresh wounds are impractical to graft from a practical

prospective, as the graft requires at least 6 weeks for development. The notion of "induced fresh wounds" (ie a blister mechanically induced by rubbing non-traumatised skin) is an option to produced standardised wounds in specific locations amenable to grafting. Siprashivili et al (2016) did select 5 chronic wounds and one induced wound for each patient. One conflicting hypothesis against grafting freshly wounded areas is that there may also be a risk that endogenous recruited epithelial progenitors would preferentially compete with the genetically corrected material. The ultimate decision has been to decide on the anatomical location of the graft on an individual basis. All areas will be considered but those that allow ease of local anaesthetic administration and wound dressings will be preferred. Sites such as the forearms will be the optimal graft site and therefore this was considered during patient selection for this study. Four out of the nine selected patients have chronic wounds affecting the forearms and therefore would be preferable for the future consideration.

Since the conclusion of this study, there have been a number of changes that would alter my optimum patient selection. Firstly, there has now been a change in the workgroup, and therefore assessments for proliferative capacity will now be sent to Madrid (CIEMAT). This will delay the date for the likely first patient to be grafted. Secondly, patients CK-07 and HS-06 have developed cutaneous SCC. This therefore would exclude them from participation. This highlights the importance of ensuring a large number of potential patients in studies of rare diseases, in which the patient cohort may succumb to both infections and cutaneous malignancies. My next options for patient recruitment would be to widen the study to include patients from other centres such as Birmingham and Solihull, with their regional EB sites designated as patient identification centres (subject to regulatory approval). The GENEGRAFT project is currently still yet to open (July 2017) and is pending approval from ANSM

the EU regulatory body. ANSM have advised that further murine studies are required in both male and female mice to ensure no downstream germline adverse effects related to the genetically corrected graft.

3.5 Summary

This chapter summarises the work performed in order to find the most suitable RDEB patient cohort for the application of a gene corrected skin equivalent graft. The main factors assessed were related to the severity of phenotype, possible predictors for the development of an immune response, likely tolerability of the graft procedure, risk of infection and possible graft site. A total of 9 patients were selected and the best three possible candidate patients were identified although since the completion of the study 2/3 developed new SCC.

Chapter 4

Predicting immune response to *ex vivo* delivered C7

4.1 Introduction

Induction of immune responses to the transgene product within donor recipients is a serious concern in the development of gene therapy clinical trials. The exact nature of response and association between transgene or target protein expression profiles and immune induction following gene transfer remains unclear. The potential for immunogenicity to the vector, transgene or newly expressed therapeutic protein can give rise to three principal concerns. First, an immediate type I hypersensitivity may occur as an immune response. Secondly, an immune response directed at the target protein may attenuate or minimise its efficacy. Thirdly, the immune response to the newly expressed protein (stimulated via gene expression of the transgene) may then induce autoimmunity to the patient's own residual native C7 protein (Schellekens, 2002).

4.1.1 The role of anti-C7 antibodies in RDEB

Antibodies targeting C7 are most commonly detected in Epidermolysis Bullosa Acquisita (EBA), an acquired auto-immune disorder characterised by mechanobullous skin fragility and similar clinical signs and symptoms as RDEB (Roenigk et al., 1971). Patients are born with normal skin and during adulthood develop IgG antibodies against C7 and anchoring fibrils in the DEJ. Epitope mapping of the C7-specific immune response in EBA patients identified the NC1 domain of C7 as the major antigenic site (Tanaka et al., 1994). Binding to the collagenous or the NC2 domain is

rarely observed in EBA (Ishii et al., 2004) although pathological blister induction is independent of targeted epitopes. Pendaries *et al* (2010) state that *in silico* prediction in RDEB samples has shown that peptide binding on different Human Leucocyte Antigen (HLA) demonstrate antigenic binding on the full length of the protein including NC1 domain, NC2 domain and the triple helical component (Pendaries et al., 2010). Experimental studies have shown that these antibodies are pathogenic. The transfer of both rabbit and human anti C7 antibodies to nude mice have resulted in the development of sub-epidermal blisters (Woodley et al., 2006, Sitaru et al., 2005). Anti-C7 antibodies have been detected in other inflammatory conditions apart from EBA such as inflammatory bowel disease and systemic lupus erythematosus, without necessarily leading to the clinical phenotype of skin blistering (Hundorfean et al., 2010, Licarete et al., 2012).

The expression or administration of a new recombinant therapeutic protein is known to initiate a humoral response (Koren et al., 2002). Remington *et al* (2009) have shown that injection of recombinant C7 in a C7 knockout mouse model led to an immune response evidenced by circulating human anti-C7 antibodies. These antibodies neither bound to the BMZ nor prevented the incorporation of newly injected human C7 into the BMZ (Remington et al., 2009).

There have now been a number of reports of a more than expected number of circulating antibodies to C7 detected in patients with RDEB prior to any treatment with gene, cell or recombinant protein therapies. Woodley *et al* (2014) have shown that 12 out of 22 RDEB patients had low levels of circulating anti-C7 autoantibodies that do not bind to the BMZ. Pendaries *et al* (2010) published the first reports of a newly validated ELISA to C7 using the full length C7 protein epitopes as well as an

ELSPOT assay to analyse the B cell and T cell responses to the full length C7 protein. Seven patients with RDEB were evaluated; only 1/7 had a positive ELISA and 3/7 had a positive ELISPOT (Pendaries et al., 2010). Two of these patients had a *COL7A1* mutation indicative of RDEB (Gen Sev) and leading to the complete absence of C7 protein evidenced on DIF and western blot. This suggests that these patients recognize C7 as a foreign antigen and points to potential rejection of C7 collagen-based gene or protein therapy. Patient 8, who showed also an elevated ELISA as well as ELISPOT score, had a healthy dizygotic twin who died at birth, suggesting that she may have developed C7-reactive T-cell clones through her contact with this protein in utero. These results highlight the necessity for assessment of an individual's immune response to C7 using *in vitro* methods, as an adjunct to the *COL7A1* mutation analysis and C7 expression levels. Tampoia *et al* (2013) have shown that 13/15 patients exhibited anti C7 antibodies. Although there have been no proven associations between levels of C7 expression and anti-C7 antibodies, both Woodley *et al* (2014) and Tampoia *et al* (2013) had commented on an association between antibody levels and disease severity scores.

However, within these cohorts of patients with RDEB there have not been any antibodies detected binding to the BMZ. The clinical significance of these antibodies is difficult to interpret. To date there has only been one case of DDEB complicated by EBA induced by autoantibodies to the NC1 domain of C7 (Hayashi et al., 2016). The mutation identified in this individual was a glycine substitution within the central triple helical domain of *COL7A1* and the mutant protein expressed was predicted to inhibit triple helix formation of wild type C7 in a dominant-negative manner. Although EBA is not known to occur in RDEB, this case highlights that *COL7A1* mutations generating mutant C7 peptides may be a risk factor for EBA in some cases.

In summary, circulating anti C7 antibodies have been detected *in vivo* in RDEB patients as well as in animal models following exposure to therapeutic C7 protein, but rarely do these antibodies bind to the DEJ. Nevertheless, all therapies for RDEB including gene therapy, cell therapy and protein therapy will involve exposure of the patient to new epitopes or domains of C7 and therefore there will be a potential to generate anti-C7 antibodies. Clearly, the presence of these antibodies should be evaluated and taken into consideration in the selection of patients for clinical trials.

4.1.2 Immune Response to genetically corrected cells

Immune response to the viral capsid of AAV vectors has been a known hurdle using this vector subtype. In contrast, lentiviral and retroviral vectors have a reduced immunogenic potential, however, the risk of immune response to transgene as well as newly expressed protein is a persistent issue. Previous studies of cutaneous gene therapy have shown that the expression of a neo-antigen in a non-tolerant host to be associated with rejection of the genetically modified tissue (Ghazizadeh et al., 2004, Dellambra et al., 2000). In *ex vivo* gene therapy studies, immune response and activation of cytotoxic T lymphocytes (CTL) is thought to be elicited by cross presentation of keratinocyte derived antigens (Heath et al., 2004). Specific transgene immune responses have been shown to develop as early as 2 weeks post transplantation using immunodeficient mouse models (Lu and Ghazizadeh, 2007). In addition to the CTL mediated destruction of gene corrected tissue following *ex vivo* gene transfer, Lu and Ghazizedah (2010) have also shown a role for the Th2 pathway and eosinophilic response. In clinical studies, however, there have been no reported incidents of adverse immune response or rejection of genetically corrected tissue in RDEB thus far. In the case of the JEB phase I study the lack of immune response was likely due to appropriate patient selection (Mavilio et al., 2006). The adult patient had

compound *LAMB3* mutations allowing residual (<5%) expression of laminin β3 polypeptide, therefore the newly grafted antigen would be unlikely to be perceived as "foreign" by the host immune system. Antibodies to the vector derived *LAMB3* in the patients' serum were not detected using western blot analysis, immunoprecipitation and immunohistochemistry. There was no evidence of an allergic or inflammatory cutaneous reaction on serial monitoring skin biopsies from the graft and donor sites. There was no detected cytotoxic or IFN-γ immune response to an autologous, *LAMB3* transduced lymphoblastoid cell line in PBMC samples at follow-up time points. This suggests that the selected patient was "tolerant" to laminin β3 polypeptide. Despite this outcome in this pioneering study in inherited skin disease, the theoretical possibility of induction of immune response to the transgene product is a definite consideration in developing clinical trial protocols for cell, gene or protein therapies that aim to increase levels of C7 at the DEJ.

When designing phase I studies, this safety aspect is of primary concern, however, the development of an immune response to transgene is also critical when aiming for increased efficacy of the proposed graft. Several other studies using *ex vivo* gene therapy have shown a correlation between antibodies against the therapeutic protein and treatment failure in animal models of liver disease (Aubert et al., 2002) and haemophilia (Van Damme et al., 2003). However, this has not been demonstrated in RDEB.

The aim of the next phase of the study was to assess the immune response to C7 *in vitro* in selected patients and to use these data to stratify those with the highest risk of developing an adverse response to the transgene product.

4.2 Methods

4.2.1 Detection of anti-C7 antibody using indirect immunofluorescence microscopy

Sera were obtained from each of the RDEB subjects at time of recruitment and sent to the Immunodermatology Laboratory at St Thomas' Hospital for analysis. Frozen sections of normal human skin (5 μm), split with 0.02M EDTA, were exposed to serial dilutions of the RDEB subjects' sera (1:10 and 1:100 in Dulbecco's PBS) for 45 minutes at 37◦C and then washed and dried in Dulbecco's PBS. FITC-labelled anti-human IgG and IgA antibodies (Dako) were diluted 1:100 in Dulbecco's PBS and applied to the sections for a further 45 minutes at 37◦C. Sections were then washed, as before, prior to mounting in Citifluor AF2 solution (TAAB Laboratories) and microscopy.

4.2.2 Detection of anti-C7 antibodies using ELISA commercial kit

Sera were obtained from each of the RDEB subjects and either used as a fresh sample or stored at -80ºC before assessment. Each sample was analysed using the MESACUP anti-C7 test kit (Medical and Biological laboratories, Japan), which is a semi-quantitative enzyme linked immunoabsorbent assay (ELISA) for the detection of antibodies to C7 in human serum. This was performed by the Immunodermatology laboratory at Guys and St Thomas' NHS Trust.

4.2.3 Detection of B-cell and T-cell response to type VII collagen using ELISPOT

The enzyme linked immunoabsorbent spot (ELISPOT) assay is a widely used assay for monitoring cell-mediated immunity, detecting antigen specific T and B cells within a population of peripheral blood mononuclear cells (PBMCs). This can detect an early

antigen directed activation of lymphocyte sub-populations at the single cell level, as a complement to the detection of circulating antibodies using ELISA. Full length recombinant C7 protein was purified from the conditioned medium of an RDEB keratinocyte cell line transduced with a *COL7A1* retroviral vector. T cells from healthy donors were stimulated with purified recombinant type VII collagen to measure the baseline reactivity to the protein. Concanavalin A (25 ng ml) was used in parallel as a positive control of T-cell reactivity. IFN-γ response was expressed as spots per well. To ensure standardisation of results compared with recruits from other centres, ELISPOT samples were sent to Paris for analysis by Professor Alain Hovnanian and his team.

4.3 Results

4.3.1 Predicted immune response to C7 based on ELISA, ELISPOT and Indirect IF

Immune response to C7 was assessed using ELISA and ELISPOT assays to detect antibodies targeted against the C7 protein. Binding of these antibodies to the BMZ, and therefore the likelihood of pathogenicity *in vivo*, was determined using indirect immunofluorescence. These assays were used in this cohort to determine the risk of developing an adverse immune response to C7, and therefore select the best candidates for further analysis and consideration for the phase 1 clinical trial.

Patient No	BEBS Score (0-100)	C7 Expression at DEJ	C7 ELISA (Full length)	C7 ELISA (NC1 + NC2)	C7 ELISPOT	Indirect IF
BH-01	**33**	++	--	--	--	--
AF-02	**24**	+	**90 (+)**	**18 (+)**	--	--
SC-03	**23**	+	**29.08 (+)**	**61 (+)**	--	--
AK-04	**27**	--	--	**34 (+)**	--	--
MJ-05	**42**	++	**26.58 (+)**	**14 (+)**	+	--
HS-06	**47.5**	++	--	--	**Borderline**	--
CK-07	**32**	--	**17.55 (+/-)**	--	--	--

Table 4.1 Summary of patients' C7 expression in the skin and anti-C7 autoantibodies in serum.

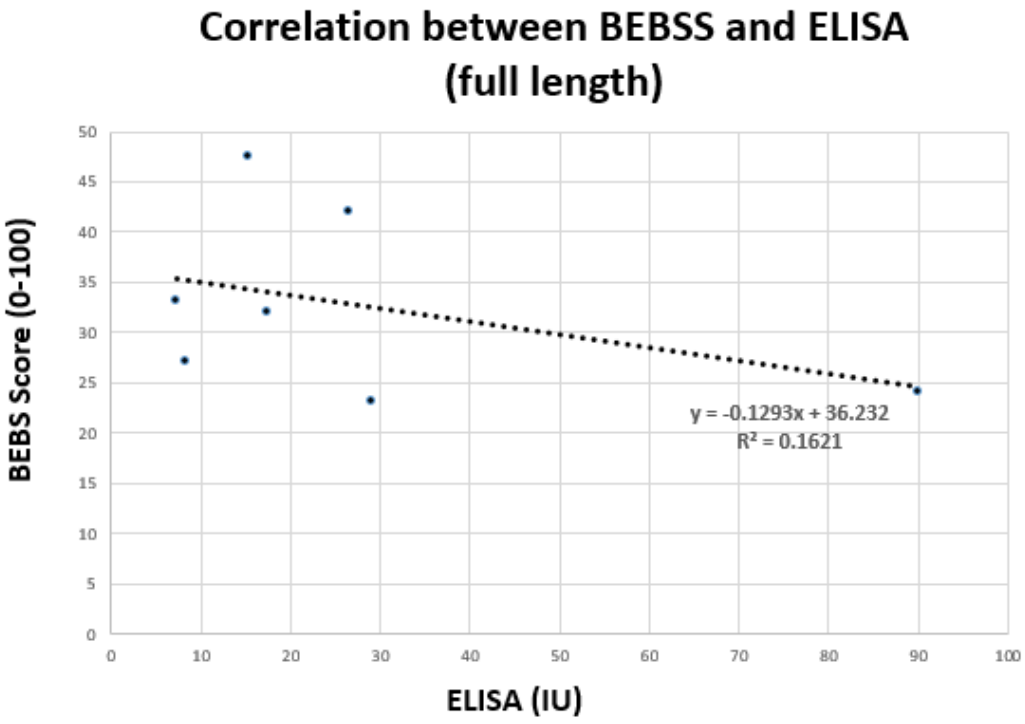
(+++) = normal
(++) = slightly reduced pattern of staining,
(+) = faint staining
(-) = complete absence

C7 expression at the DEJ was determined by immunofluorescence staining of cryostat sections with an anti-NC1 antibody. ELISA was performed with a commercially available Mesacup kit (Medical and Biological laboratories, Japan) that uses a mixture of immobilized NC1 and NC 2 domains as the target substrate (<6 U = normal). A further assay used full-length, recombinant human C7 as the target substrate (C7 ELISA full length, < 20 U = normal dependent on parallel control). ELISPOT assay analyses B- and T-cell responses to full length C7. Indirect immunofluorescence was used to assess the binding of circulating IgG antibodies to salt split skin. 4/7 (02, 03, 04, 05) patients showed positive anti-C7 antibodies in their sera using a commercially available ELISA kit which targets both the NC1 and NC2 epitopes. 4/7 (02, 03, 05, 07) also showed positive anti-C7 antibodies in their sera using ELISA assay targeting full length recombinant C7 protein.

Abbreviations : BEBS = Birmingham Epidermolysis Bullosa Score, IF = Immunofluorescence, NC-1 = non-collagenous domain 1, NC2 = non-collagenous domain 2.

4.3.2 Correlation of immune response with markers of disease severity

Woodley *et al* (2014) and Tampoeia *et al* (2013) have both shown a correlation between disease severity scores and predicted immune response to C7 (Woodley et al., 2014, Tampoia et al., 2013), with those with a higher BEBS score having a greater predicted immune response. My data shows a similar association but not significantly. The limitation in most cases is the small numbers of cases analysed, as well as the objective measure of disease severity. I used the BEBS score which takes into account eleven items, including area of damaged skin, involvement of nails, mouth, eyes, larynx and oesophagus, scarring of hands, skin cancer, chronic wounds, alopecia and nutritional compromise. The method is simple, easy to use and applicable to all types of EB but the limitations are that only one item takes into account disease activity. Although BEBS is a validated objective measure of diseases severity, it can be difficult ascertain subtle or transient changes in disease activity versus chronic scarring and contractures. Other disease scores such as the Epidermolysis Bullosa Disease Activity and Scarring Index (EBDASI) score has the ability to distinguish activity scores that are responsive to therapy separately from damage (Loh et al., 2014). The iscorEB (Instrument for Scoring Clinical Outcome of Research for Epidermolysis Bullosa), in contrast to previous scoring models, includes not only the perceptions of disease severity of EB health care professionals, but also patient and parent perception of disease severity. Perhaps other scores such as EBDASI and iscorEB would be useful to run in parallel for the assessment of immune response to C7. At the time of study design the BEBS score was the only validated published severity score for EB available.



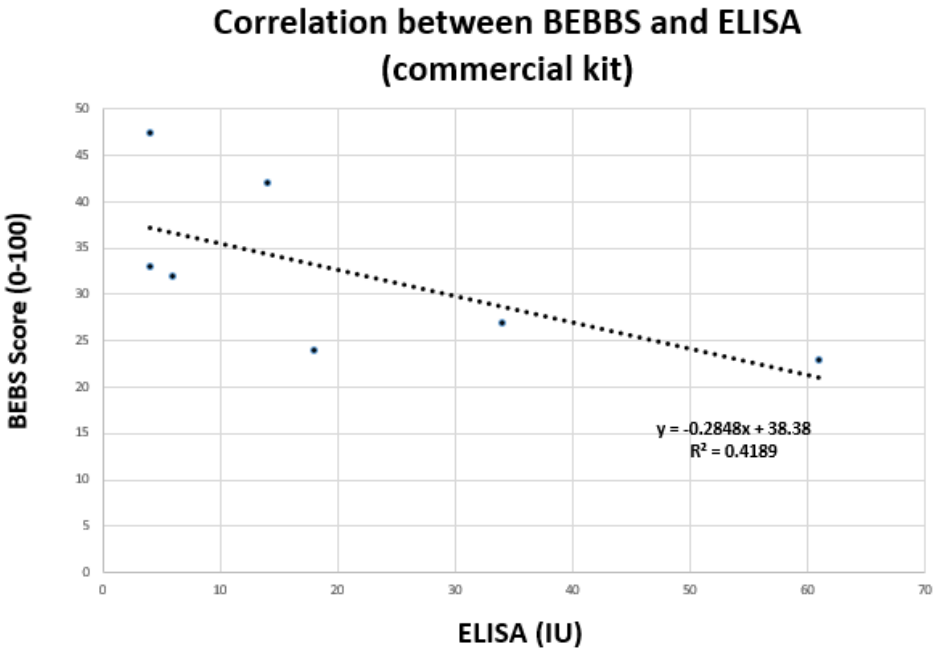


Figure 4-1 Correlation between BEBS scores and ELISA assays. Correlation is determined by Spearman rank coefficient, r = 0.16 and r = 0.41.

Abbreviations : BEBS = Birmingham Epidermolysis Bullosa Severity Score, ELISA = Enzyme linked immunosorbent assay

4.4 Discussion

This study has shown that circulating anti-C7 antibodies have been detected using ELISA and to a lesser extent ELISPOT analysis in a significant number of study subjects (See Table 4.2). This observation has been validated by other groups (Woodley et al., 2014, Tampoia et al., 2013) also demonstrating that a higher than expected proportion of patients with RDEB have *de novo* circulating antibodies that are non-pathogenic through lack of binding to the BMZ. Woodley *et al* (2014) reported that 12/22 of RDEB patients exhibited anti-C7 antibodies, but none showed pathogenic binding to BMZ. The finding of anti-C7 antibodies did not correlate with expression of C7 in patients' skin, however a correlation between antibodies and BEBS score was detected by both Woodley *et al* (2014) and Tampoia *et al* (2013). Perhaps an explanation for this observation could be an altered recognition of "self" due to altered protein synthesis secondary to epigenetic mechanisms affecting cDNA gene expression. Epigenetic mechanisms primarily consist of DNA methylation, histone modifications and small non-coding RNA transcripts, which may then lead to the development of autoimmunity. The correlation between anti-C7 antibodies as well as auto-antibodies to other constituents of the BMZ such as BP180 and BP230 could suggest "epitope spreading" as an underlying phenomenon, with recruitment and activation of autoreactive T-lymphocytes specific for secondary epitopes (Tampoia et al., 2013). Previous exposure to neo-antigen following previous fibroblast cell therapy may be relevant in my own cohort. 4/7 study subjects (02,03,05,07) had received previous intradermal allogeneic fibroblasts at least six months before study inclusion and interestingly all of these patients had detectable anti-C7 antibodies using either ELISA assay and one of them had a positive ELISPOT. Of these patients, 2/7 patients had received allogeneic fibroblasts at more than one time point with no development

of detectable clinical adverse immune response to the therapy. In terms of efficacy, MJ-05 reported improved response to the cell therapy after second administration of cells, therefore suggesting that the development of anti-C7 antibodies did not play a role in a reduction of therapeutic efficacy. The high proportion of positive C7 auto-antibodies using ELISA in this and other cohorts does minimise its usefulness and validity as a screening tool for *ex vivo* gene therapy clinical trials. These findings have been substantiated by the recently published EBSTEM study, in which 9/10 children with RDEB had positive anti-C7 antibodies detected using a commercially available ELISA kit (Petrof et al., 2015). In this cohort there were no sera binding to the DEJ using IIF and also there were no changes in ELISA or IIF following treatment with intravenous allogeneic mesenchymal stromal cell therapy. Based on our findings as well as others, a positive ELISA should only be used as an inclusion or exclusion criteria if a corresponding IIF analysis shows binding of the antibodies to skin. The ELISPOT assay appears to be a more specific assay in this study cohort. Only one patient had a positive ELISPOT compared to 4/7 patients with positive ELISA. None of the patients showed pathogenic binding of these autoantibodies to the BMZ. There have no experimental animal models assessing whether these antibodies have a pathogenic role in RDEB. There have been several reported cases of adult patients with RDEB who developed patches of clinically normal skin that never blister due to spontaneous *COL7A1* gene correction in a subpopulation of keratinocytes (not fibroblasts) (Almaani et al., 2010, Tolar et al., 2014). In one case exposure to the newly recognised C7 protein led to the development of auto-antibodies, but with no pathological binding to the BMZ and no reported clinical sequelae. Based on clinical studies it would appear that these antibodies are not clinically relevant, however, the effect on graft outcome and risk of rejection has yet to be demonstrated. In terms of

grafting the first patient, it may be preferable to select a subject with negative ELISPOT and Indirect IMF in the first instance as a definitive criterion. The positive finding of anti-C7 antibodies as detected by ELISA appears to be a finding more common than previously anticipated in untreated RDEB patients and therefore should not represent an absolute exclusion criterion: this observation has important implications for defining inclusion/exclusion criteria in other clinical trials in the future.

4.5 Summary

This chapter summarises the findings related to a predicted immune response to exogenous C7 protein in 7 of the selected patients recruited in for the EBGEN study. 4/7 patients had a positive ELISA to C7 and only one patient had a positive ELISPOT. None of the samples showed binding to the BMZ suggesting likely pathogenicity. This raises the question for the necessary investigations required to predict a likely immune response.

Chapter 5

Patient perspectives towards gene therapy clinical trials

5.1 Patient and public involvement in clinical trials

Patient and public involvement (PPI) in the design and development of clinical trials is critical both to ensure that the primary needs of the patient community are addressed but also to guarantee adequate levels of recruitment (Gamble et al., 2014, Boote et al., 2011). The research community has been increasingly made aware that patients and the public are stakeholders in clinical trials and should be directly involved in trial design (Mullins et al., 2014). The UK Department of Health (2006) Best Research for Best Health guidelines state that PPI must be included in all stages of the research process including priority setting, defining research outcomes, selecting research methodology, patient recruitment, interpretation of findings and dissemination of results (Evans, 2006). This is particularly important in rare diseases such as RDEB. Studies examining the challenges of planning clinical trials for other rare genetic disorders such as phenylketunoria (PKU) have highlighted issues including the relatively small pool of known patients with specific phenotypes, the logistical issues including the long distances to travel to the nearest disease centre, and the significant burden of disease leading to dependence on carers and family leading to psychological burdens and lack of hope for curative treatments (DeWard et al., 2014). Davila-Seijo *et al* (2014) have also highlighted that most current randomised clinical trials for RDEB do not match the research needs as perceived by clinicians and patients (Davila-Seijo et al., 2014), as researchers appeared to be dedicated to more high-risk high

technology approaches such as bone marrow transplant whereas patients research needs were mainly related to symptoms control and more "everyday problems". Phase I proof of principle studies such as the GENEGRAFT study aimed at developing long-term curative approaches for the disease may not be perceived to be a research priority by patients. Patient and public involvement at an early stage is therefore critical to ensure patient engagement in these early studies and tailor the protocol to patient needs as much as possible.

PPI can be undertaken using patient questionnaires, focus groups or the involvement of patients or patient organisations in review of trial documents and protocols. The main areas for patient involvement related to the GENEGRAFT study would be in relation to the location of the grafted skin equivalent and also the outcome measures that should be assessed. Methodology and outcomes based on relevance to patients would lead to findings having a greater impact on patient care. The key questions areas that I wished to explore were related to

1. Awareness of current gene therapy clinical trials for RDEB.
2. Willingness to proceed with grafting procedure for the purposes of a clinical trial.
3. Favoured locations of wounds requiring grafting.
4. Favoured areas of grafting (ie. blistered versus unblistered sites).
5. Willingness to travel outside of the UK for the purposes of a clinical trial.

5.2 Electronic patient survey

An electronic patient survey was designed and reviewed by the clinical lead for the EB service (see Appendix 2). The survey entitled "A survey of opinions regarding

gene therapy options for patients with RDEB" received approval from the Research and Development division at Guys and St Thomas' NHS Trust in September 2014 (RJ114/N254).

5.3 Survey outcomes

Following consultation with the clinical specialist nursing team 20 patients with RDEB were identified with access to email and a current email address. There were 12 respondents to the survey sent out using the server "survey monkey". All results were anonymised and data stored in accordance with the Guys and St Thomas' Hospital NHS Foundation Trust Data Protection guidance. All respondents had a confirmed diagnosis of RDEB and were under the clinical care of GSTT. The majority were aged under 30 (n=10/12) and the male/female ratio was 7/5 (M/F).

Table 5.1. Abridged results of electronic survey of patients' opinions regarding options for gene therapy clinical trials

	YES	NO	NOT SURE
HAVE YOU EVER TAKEN PART IN A CLINICAL TRIAL?	9 (75%)	3 (25%)	-
HAVE YOU HEARD OF A NEW THERAPY FOR RDEB CALLED “GENE THERAPY”?	7 (58.3%)	3 (25%)	2 (16.6%)
WOULD YOU CONSIDER DISCUSSING TAKING PART IN A GENE THERAPY CLINICAL TRIAL?	8 (66.6%)	3 (25%)	1 (8.33%)
WOULD YOU CONSIDER HAVING A SKIN GRAFT OF GENETICALLY CORRECTED CELLS?	6 (50%)	2 (16.6%)	4 (33.3%)
WOULD YOU BE WILLING TO HAVE A SKIN GRAFT OF ON AN AREA OF YOUR BODY THAT WAS UNBLISTERED?	4(33.3%)	3(25%)	5(41.67%)
WOULD YOU BE WILLING TO HAVE A SKIN GRAFT OF AN AREA OF YOUR BODY THAT WAS RECENTLY BLISTERED?	4(33.3%)	3(25%)	5(41.67%)
WOULD YOU BE WILLING TO HAVE A SKIN GRAFT ON AN AREA OF YOUR BODY THAT WAS CHRONICALLY WOUNDED (IE >6 MONTHS)?	5(41.37%)	3(25%)	4(33.3%)
WOULD YOU CONSIDER HAVING AN INJECTION OF GENETICALLY CORRECTED CELLS INTO A WOUND?	6(50%)	3(25%)	3(25%)
WOULD YOU BE WILLING TO TRAVEL OUTSIDE OF THE UK TO RECEIVE A SKIN GRAFT OR INJECTION OF CORRECTED CELLS AS PART OF A CLINICAL TRIAL?	9(81.2%)	2(18.18%)	--

5.4 Discussion

The results presented in Table 5.1 highlight both positive and negative patient perspectives. The majority of respondents had already taken part in a clinical trial previously which had coloured their perspectives on entering another trial. Over half of those questioned would consider taking part in a gene therapy clinical trial in the future and similar numbers would consider intradermal injections as well as a genetically corrected graft. This was an unexpected finding, as the grafting procedure is a more invasive procedure and I would have expected the majority to prefer the intradermal approach. This may reflect the strong willingness of the patient population to further the research field in desperate hopes for a cure. Another factor may be that most of the patients who had been involved in a previous clinical trial would have received intradermal allogeneic fibroblasts and therefore would be aware of the associated pain. One of the main aims of this survey was to help guide the decision regarding graft site however there did not appear to be significant differences in patient preferences between unblistered sites, recently blistered sites or chronically wounded sites, with only a slight majority favouring chronically blistered sites (33.3% vs 33.3% vs 41.7%). The significant majority of patients would be willing to travel outside of the UK in order to receive treatment as part of a clinical trial, and also outside of the EU if necessary. One limitation of the survey was that the duration of time outside of the UK was not specified. A protocol involving grafting multiple sites would necessitate a significant period of time outside of the UK with up to 21 days to ensure direct surgical wound reviews and dressing reviews in a sterile monitored environment.

One of the most interesting aspects of this survey were the responses to the open-ended questions (Appendix 1). There are clear limitations in the interpretation of qualitative

data in such a small number of respondents. The reasons given for their decision to participate (or not) in a gene therapy clinical trial involving a skin graft clearly showed both points of view. Four of the seven responses were positive in relation to the study and showed a somewhat altruistic desire to further clinical trials in RDEB with comments such as "…I am keen and willing to support any initiatives that may be of benefit, be that for me 'today' or others in the future…" and "..I know it's not a cure yet but if one piece of skin is corrected maybe with more trials more of my affected skin can be corrected". Three responses were perceived to be negative in regards to participation and are related to previous experiences with grafting and the expected reaction to an invasive procedure such as grafting, with comments such as "..having had my hand operated on and a skin graft done on my leg… I can see the negative effects the procedure can create on the donor site and also issues that can arise with the newly placed skin".

5.5 Summary

In summary, this brief survey has highlighted the perceived value of gene therapy clinical trials within my own cohort of patients and emphasised both the optimism and valid trepidations of such ventures. The most important finding is that, despite concerns, the majority of patients would be willing to consider involvement in a trial involving gene therapy, and also consider travelling within the EU to participate.

Chapter 6

Pre-clinical development of phase I clinical trial of transduced primary RDEB fibroblasts using a SIN lentiviral vector

6.1 Introduction

Pre-clinical studies have thus far aimed to increase levels of C7 at the DEJ via gene replacement strategies, cell therapies, protein therapies or drug therapies. The rationale for the use of gene modified fibroblasts in RDEB is based on the earlier clinical studies that used intradermal allogeneic and non-gene modified autologous fibroblasts to treat localized wounded areas (Petrof et al., 2013, Wong et al., 2008). Allogeneic fibroblasts have been shown to increase patients' own levels of C7 and improve skin integrity for up to 9 months following a single injection (Wong et al., 2008, Nagy et al., 2011). These studies have shown promising results but are limited. Wong *et al* used fluorescent in situ hybridization (FISH) for the detection of donor Y-positive cells in a female recipient and demonstrated the clearance of fibroblasts within 2 weeks following intradermal injection. The use of gene corrected autologous cells would therefore offer a complementary approach that could feasibly offer longer term correction and reversion of C7 at the DEJ, but also diminish the need for immunosuppressive medication. *Ex vivo* gene therapy pre-clinical studies have shown that viral vectors, either retroviral or lentiviral vectors carrying the *COL7A1* cDNA, are able to efficiently transduce patients' own fibroblasts. *In vitro* and murine models have shown that the gene corrected cells were able to secrete C7 at the DEJ and reduce blistering tendency. The rationale for using autologous gene-modified fibroblasts is that (a) these cells will persist for far longer than the allogeneic cells and (b) they will generate wild-type and not mutant C7. Goto *et al* (2006) have shown that gene

corrected murine fibroblasts alone is sufficient to correct the DEB phenotype (Goto et al., 2006). The major challenge is to translate this to a phase I study and provide proof of principle data necessary for larger studies.

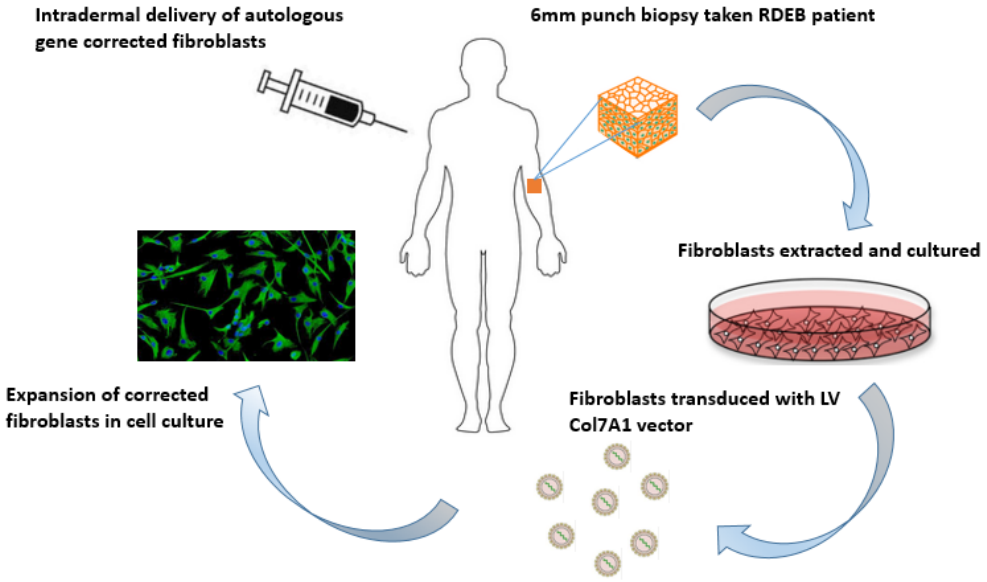


Figure 6.1. Schematic showing the rationale for the use of gene corrected intradermal fibroblasts

6.1.1 SIN *COL7A1* lentiviral vector for the transduction of RDEB fibroblasts

In order to improve the safety of gene therapy using retroviral vector technology, newer self-inactivating (SIN) retroviral and lentiviral vectors have been developed. SIN vectors have a 400bp deletion in the U3 region within the 3' LTR of the transfer vector (designated ΔU3) which abolishes full-length vector RNA in transduced cells following reverse transcription (Thornhill et al., 2008). Based on these studies a viral vector was developed under the guidance of the Institute of Child Health, based on a vector construct previously used for previous clinical trials for treatment Wiskott-Aldrich Syndrome (Galy and Thrasher, 2011, Scaramuzza et al., 2012), ADA-SCID (Gaspar et al., 2004) and Chronic Granulomatous Disease (Grez et al., 2010). The

vector backbone is based on the 3rd generation SIN lentiviral vectors first published by Dull *et al* in 1998 (Dull et al., 1998b). Packaging constructs are deleted of all HIV accessory proteins (vpu, vpr, nef, vif) and Tat. The conditional packaging system segregates gag/pol and rev genes. The transfer vector (pCCL SIN cPPTmtWPRE) encodes a modified cDNA *COL7A1* under control of a human PGK promoter element. An internal human cellular phosphoglycerate kinase (PGK) promoter element (Ginn et al., 2010, Huston et al., 2011) is incorporated in pCCL-PGK-*COL7A1* vector. The PGK promoter has been previously shown to drive *COL7A1* gene expression in RDEB fibroblasts as well as in keratinocytes using SIN retroviral vector systems (Pendaries et al., 2010). Incorporating human promoter elements has been shown to reduce the risk of unwanted activation of genes adjacent to the integration site (Modlich et al., 2006) and predisposition to toward activation near to transcriptional start sites (Zufferey et al., 1998a, Montini et al., 2006). The vector encodes the HIV-1 central polypurine tract element (cPPT) and mutated Woodchuck hepatitis post-regulatory element (WPRE) which has been shown to enhance transgene expression (Zufferey et al., 1999). The vector is pseudotyped with the Vesicular Stomatitis Virus (VSV) envelope, which exhibits strong multi-lineage tropism. The Rev responsive element (RRE) maintained in the gag/pol plasmid ensures gag/pol gene expression is Rev dependent.

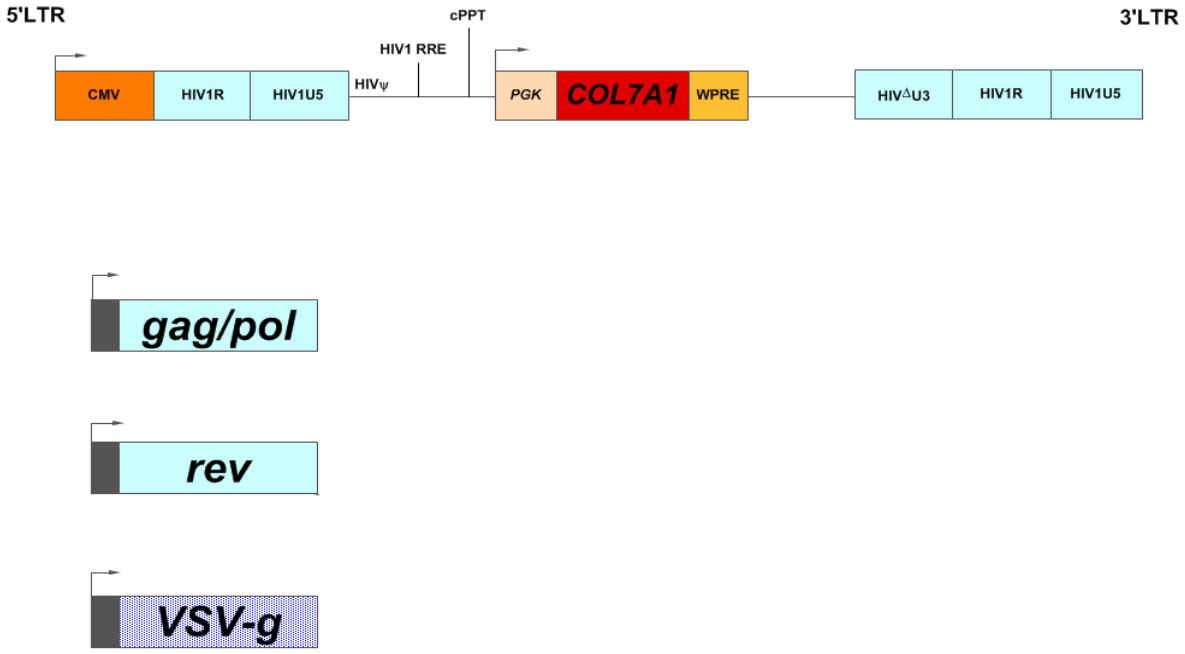


Figure 6.2 Diagram showing SIN pCCL-PGK-COL7 construct.

The transfer plasmid is shown above with the modified *COL7A1* cDNA under control of the human PGK promoter element. The packaging plamids in blue segregate gag/pol and rev and the envelope plamid on the bottom row is pseudotyped with VSV.

6.1.2 Aims

The aim of this part of the thesis was to prepare the pre-clinical data and rationale necessary for a phase I clinical trial of intradermal autologous *pCCL-PGK-COL7A1* gene corrected autologous fibroblasts in patients with RDEB. The specific aim was to assess the transduction efficiency and levels of gene replacement of a SIN pCCL-PGK-COL7 vector in primary RDEB fibroblasts.

6.2 Methods

6.2.1 Viral vector production

Viral vector production was performed by the team at the Institute of Child Health. The production of lentiviral vector involves four stages: (1) Seeding of master cell bank 293T cells at optimal density, subculture and expansion (2) Transient transfection

with the 4-plasmid lentiviral packaging system, (3) Harvesting of viral supernatant containing the lentivirus pCCL-PGK-COL7A1 and (4) Concentration and aliquoting the lentivirus.

6.2.2 Fibroblast transduction with LV vector

Primary RDEB fibroblasts were extracted from skin biopsies using the explant method (Normand and Karasek, 1995) cultured to generate sufficient numbers and transduced using the SIN LV vector at the earliest passage possible. The main aim was to achieve transduction efficiencies approaching 10-20%, aiming for less than mean 1 copy/cell. Multiplicity of Infection (MOI) is a standardised parameter for the prediction of gene transfer events (Andreadis et al., 2000). MOI was based on the following equation:

$$\text{MOI} = \frac{(\text{Viral titre}/1000) \times \text{volume viral supernatant (ul)}}{\text{Cell number}}$$

Cell number and volume of supernatant added was adjusted based on the viral titre of each supernatant batch. Cell toxicity is likely at volumes over 10% supernatant volume: media volume. Based on cell number used, either 6 well, 24 well or T25 flasks were used. Untransduced fibroblasts were cultured alongside the transduced cells and subjected to parallel culture conditions and passage number. Vector titre was assessed by seeding of 10^5 293T cells in 24 well plates followed the next day by exposure to serial dilutions of vector stock. After 72 hours, vector copy number was quantified by qPCR targeting HIV psi and subsequently calculating virus copies per cell based on a control albumin gene. The vector titre can be estimated across a range of virus volumes (0.0032ul to 10.0ul) but the estimate of stable transducing units is considered most accurate when 1-10% of cells are transduced (0.01-0.1 copies per cell). A control cell line known to encode 3 lentiviral copies per cell provided a reading of 2.6 copies per

cell in this assay and was used as an internal control. Copy number in untransduced fibroblasts was 0%.

6.2.3 Migration assay

The Oris™ Cell Migration Assay (AMS Biotechnologies UK Ltd, Abingdon, UK) is a reproducible, sensitive, and flexible assay that can be used to monitor cell migration. Formatted for a 96-well plate, the assay utilizes Oris™ Cell Seeding Stoppers made from a medical-grade silicone to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2mm diameter unseeded region in the centre of each well, i.e., the detection zone, into which the seeded cells may then migrate. The Oris™ Detection Mask is applied to the plate bottom and restricts visualization to the detection zones, allowing only cells that have migrated to be detected. Readout can be performed by microscopy or use of a microplate reader.

The Oris plate was first placed in the tissue culture hood in order to normalise the temperature and then the seeding stoppers placed firmly into the 96 well plate. Fibroblasts were then trypsinised, counted and plated at a density of 5×10^4 per well in 100µl. The cells were then incubated at 37ºC for 24 hours in order to allow cell attachment. The cell stoppers were then carefully removed, the media removed and then the cells washed very gently with PBS. Those wells designated t=0 were then fixed and imaging analysis performed. The rest of the cells were then left for 30 hours in order to assess cellular migration into the detection zone. After 30 hours, the cells were washed and then immunocytochemistry performed using according to methods previously described.

6.3 Results

This chapter describes the results of experiments performed to assess the transduction efficiency of the pCCL-PGK-COL7 vector in primary RDEB fibroblasts.

6.3.1 The pCCL-PGK-COL7 fibroblasts express moderate amounts of type VII collagen

The expression levels of C7 were assessed in pCCL-PGK-COL7 fibroblasts using immunofluorescence microscopy and Western blot techniques. There was a discernible increase in levels of C7 in RDEB fibroblasts up to 2 months post transduction with the pCCL-PGK-COL7 LV vector, despite cells going through at least five passages. There was no evidence of gene silencing up to 2 months post transduction. There were higher levels of C7 staining using a protocol of transduction with MOI approaching 20.

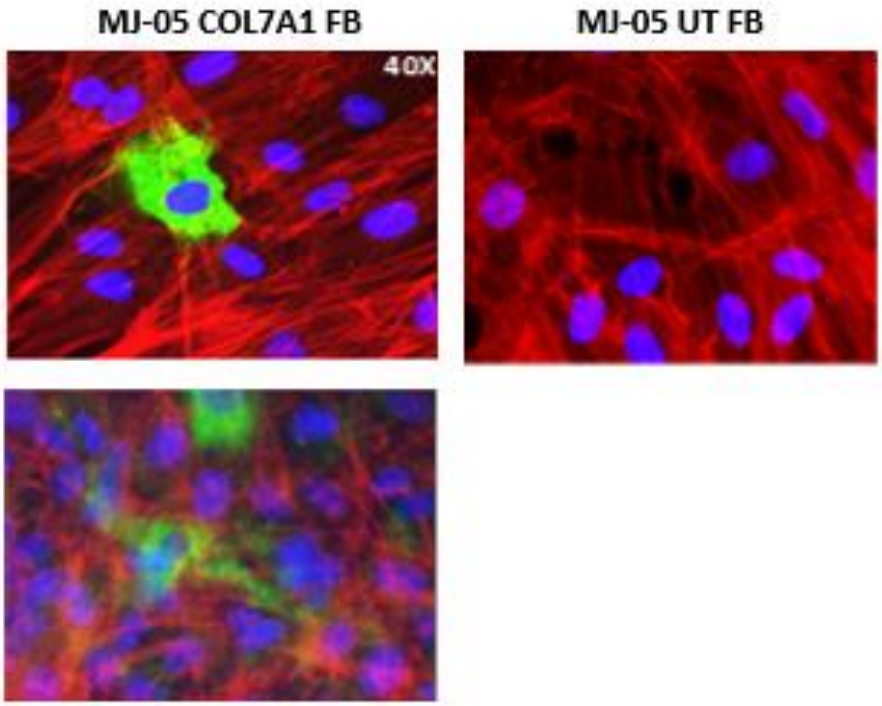


Figure 6-3 Immunocytochemistry showing C7 expression in RDEB fibroblasts transduced with LV vector

Primary FBs were stained with COL7A1 antibody (LH7.2 7.2 Sigma) (green), cell actin-filaments were stained with phalloidin (red) and cell nuclei were stained with DAPI (blue). Transduced fibroblasts show C7 expression at d3 post transduction. Scale bar = 10μm.

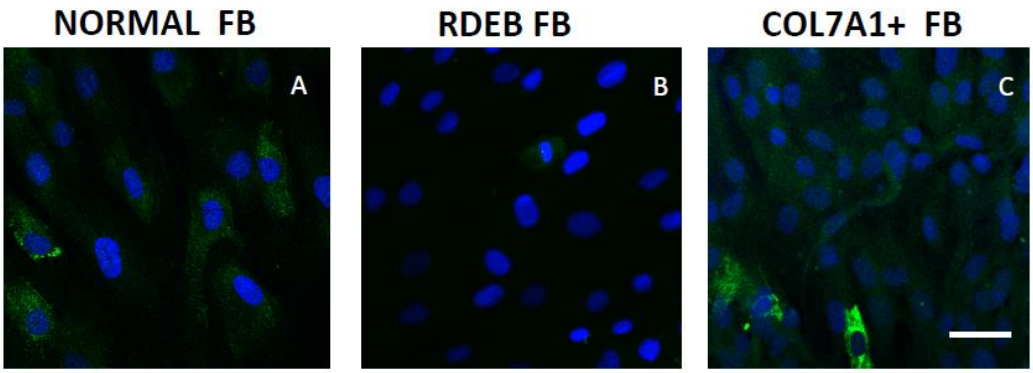


Figure 6-4 C7 Expression in cultured fibroblast showing the effect of transduction with pCCL-PGK-COL7 LV vector

Increase C7 immunolabelling is seen in cultured primary RDEB fibroblasts 2 months following incubation with pCCL-PGK-COL7 LV vector. (A)Primary wild type Fibroblasts, (B) Primary RDEB fibroblasts, (C) RDEB fibroblasts 60 days following incubation with SIN LV *COL7A1* vector, Multiplicity of Infection = 7, (D) RDEB fibroblasts 60 days following incubation with SIN LV *COL7A1* vector, Multiplicity of Infection = 17.4. Scale bar = 25µm.

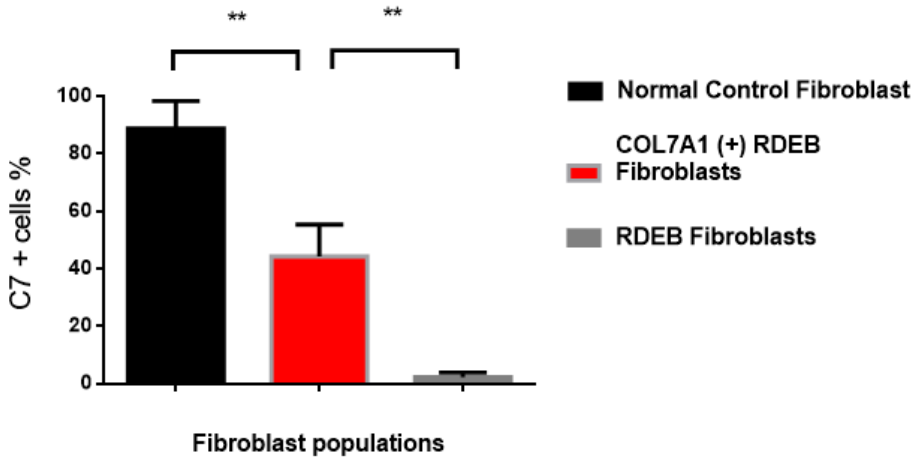


Figure 6-5 Comparison of RDEB fibroblasts before and after gene correction and normal control by immunofluorescence staining

Percentage of C7 positive cells as calculated per three independent fields of 100 DAPI positive cells. Values expressed as mean +/- standard Deviation. A significant difference in C7 expression levels was seen in cultured primary RDEB fibroblasts 2 months following transduction with pCCL-PGK-COL7 LV vector. Unpaired two-tailed Students' t-test, ** $p<0.01$

The increased level of C7 protein in transduced cells was also demonstrated using Western blot assay in both keratinocytes and fibroblasts. C7 protein expression was

determined once sufficient cell numbers had been cultured (at least 2 x 10^6). C7 represents less than 0.001% of total protein extracted from skin, and therefore it was not possible to detect C7 using Western blot at early stages post-transduction as sufficient cell numbers were required. I could therefore not compare protein extraction at earlier time points. As discussed previously, keratinocytes secrete far higher quantities of C7 than dermal fibroblasts and therefore higher protein lysate concentration was required to adequately show a band corresponding to 290kD. The levels of protein expression were quantified using Image J software (NIH image) and show that transduction using pCCL-PGK-COL7 LV vector led to protein levels a more than two fold change in levels of C7 protein compared to baseline levels.

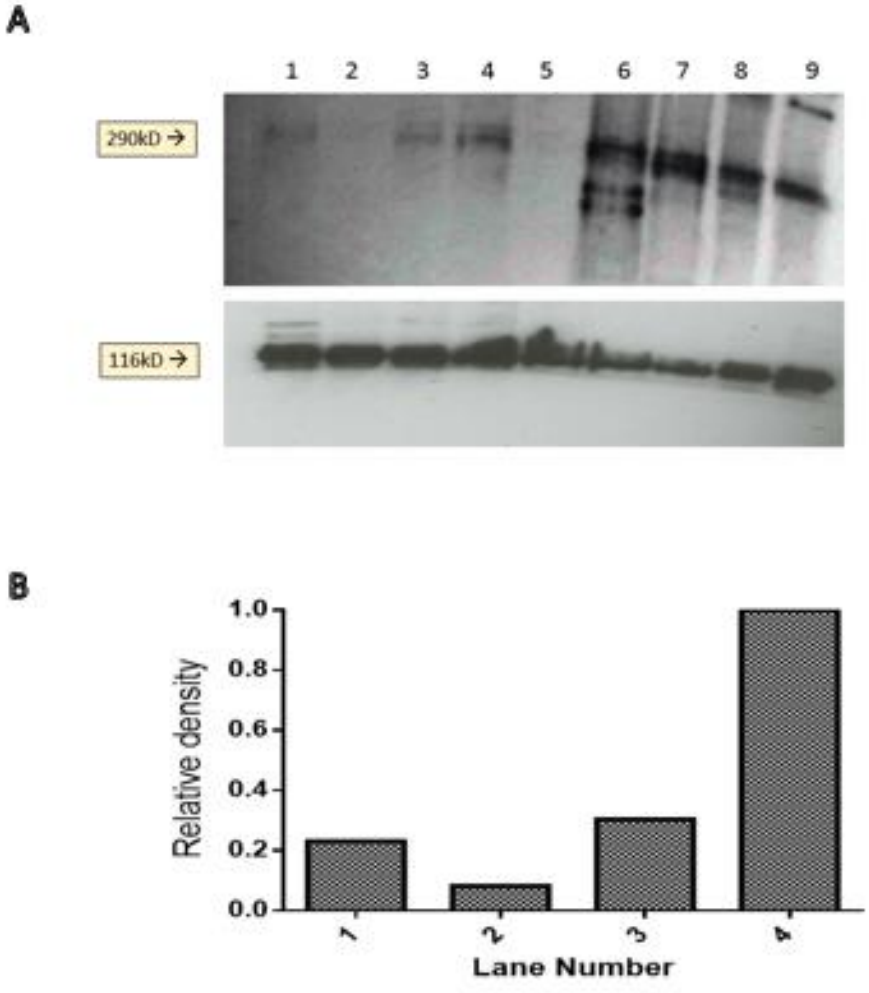


Figure 6-6 Detection of C7 expression in RDEB fibroblasts

Protein was extracted from cell lysates of fibroblasts and keratinocyte cultured cells 60 days after transduction with pCCL-PGK-COL7 LV vector (MOI 20). 50µg of protein were subjected to 4–15% SDS-PAGE followed by immunoblot analysis using an anti-NC1 antibody.(A) Lane 1: Day 24 (MOI 10) post- transduced fibroblasts; Lane 2: untransduced RDEB fibroblasts; Lane 3: day-24 (MOI 20) post-transduced fibroblasts); Lane 4: wild type control fibroblasts; Lane 5: untransduced RDEB keratinocytes, Lane 6 and 7: day-24 (MOI 10) transduced keratinocytes, Lane 8 day 24 (MOI 20) transduced keratinocytes, Lane 9 : wild type control keratinocytes. Protein ladder is shown on the left side of the blot. The position of full-length 290 kDa C7 and loading control marker 116kD vinculin is indicated. (B) The relative quantities of C7 protein in fibroblast cell cultures only (Lanes 1-4) were calculated using Image J software and expressed as a relative proportion.

6.3.2 MOI of pCCL-PGK-COL7 vector

In order to develop the most efficient transduction protocol for this cell type, optimal MOIs were assessed by observing fibroblast proliferation rates and assessment of cell

death. Based on the results outlined, it is clear that MOI > 25 leads to increased levels of cell toxicity and increased cell death.

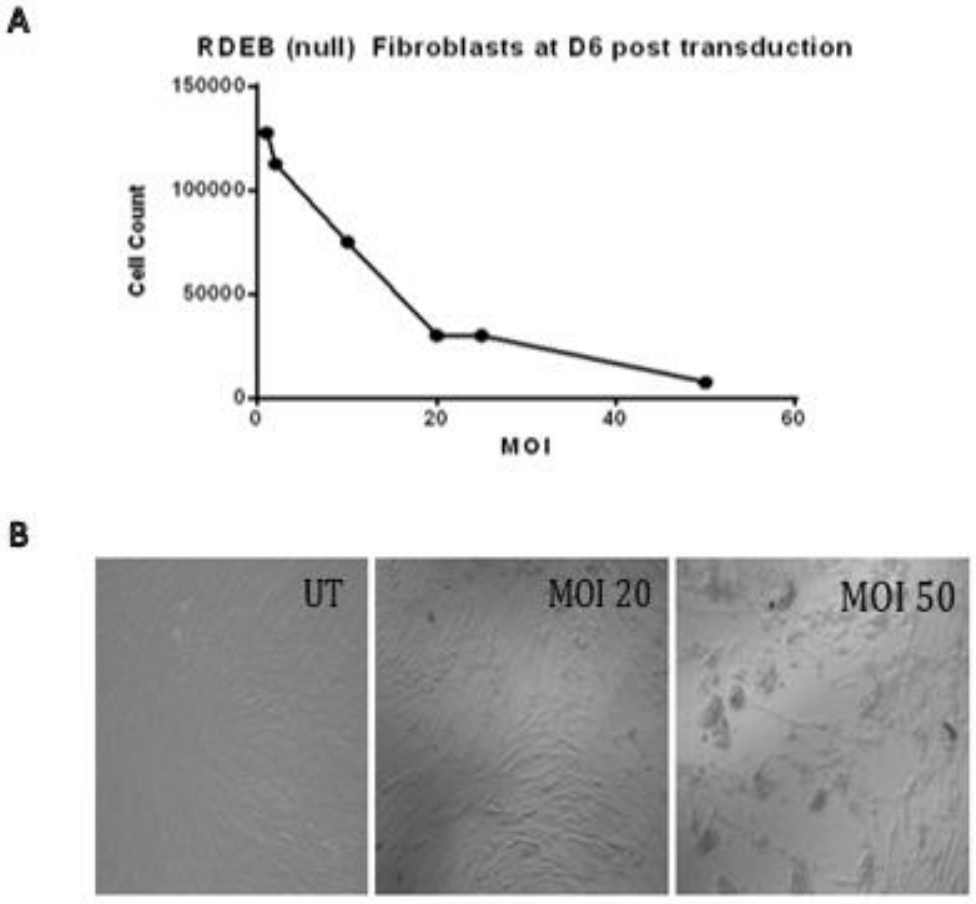


Figure 6-7 Primary RDEB Fibroblasts at Day 0 (UT), and at Day 6 post transduction at MOI 20 and MOI 50.

The morphology of gene corrected fibroblasts is increasingly altered with evidence of increasing cell death (cell shrinkage with evidence of cellular debris) with increasing MOI

6.3.2 Transduction efficiency assessment following gene transfer

The transduction efficiency of viral vectors is typically assessed using qPCR methods in order to assess viral copy number as a percentage of cells transduced, and has been a validated method following gene transfer in haematopoietic stem cells (Hanawa et al., 2004). However, in these experiments the qPCR transduction efficiency did not correlate directly with the results generated using FACS analysis. Copy number assessment on transduced fibroblasts harvested on D3 showed only 0.009 copies per cell (9% transduction). Results showed reconstitution of C7 in RDEB fibroblasts transduced with the pCCL-PGK-*COL7A1* vector with the initial transduction efficiency assessed using FACS analysis at ~21% at day-3 reflecting transient gene expression, and by 24 days, stabilising at ~9% in keeping with copy number data. Inaccuracies of qPCR in transduction efficiency assessment has been observed in other studies (Klein et al., 2000) and therefore both assays should be used to assess efficient gene transfer *in vitro*.

Source of Fibroblasts	**Days post transduction**	**Copy number**	
		MOI = 10	**MOI = 20**
EB-CK	**Day 24**	**0.09**	**0.09**
EB-8	**Day 24**	**0.071**	**0.088**

Table 6.1. Copy number assessment of primary RDEB fibroblasts following varying MOI of vector

To further determine the level of C7 expression in transduced fibroblasts using the current transduction protocol I used FACS analysis to accurately count the number of cells expressing C7 as evidenced by staining with LH 7.2 monoclonal antibody. Flow cytometry performed on primary fibroblasts was assessed using the monoclonal antibody (LH 7.2) to the NC1 domain of the type VII collagen protein. Culture conditions were standardised ensuring that all cell types were at a similar passage and had been grown in similar conditions. Using flow cytometry, the transduction efficacy ranged from 18-22% at day-3 and 9-10% at day 24, with a mean pro-viral copy number, using qPCR, of 0.063-0.09 at day 3.

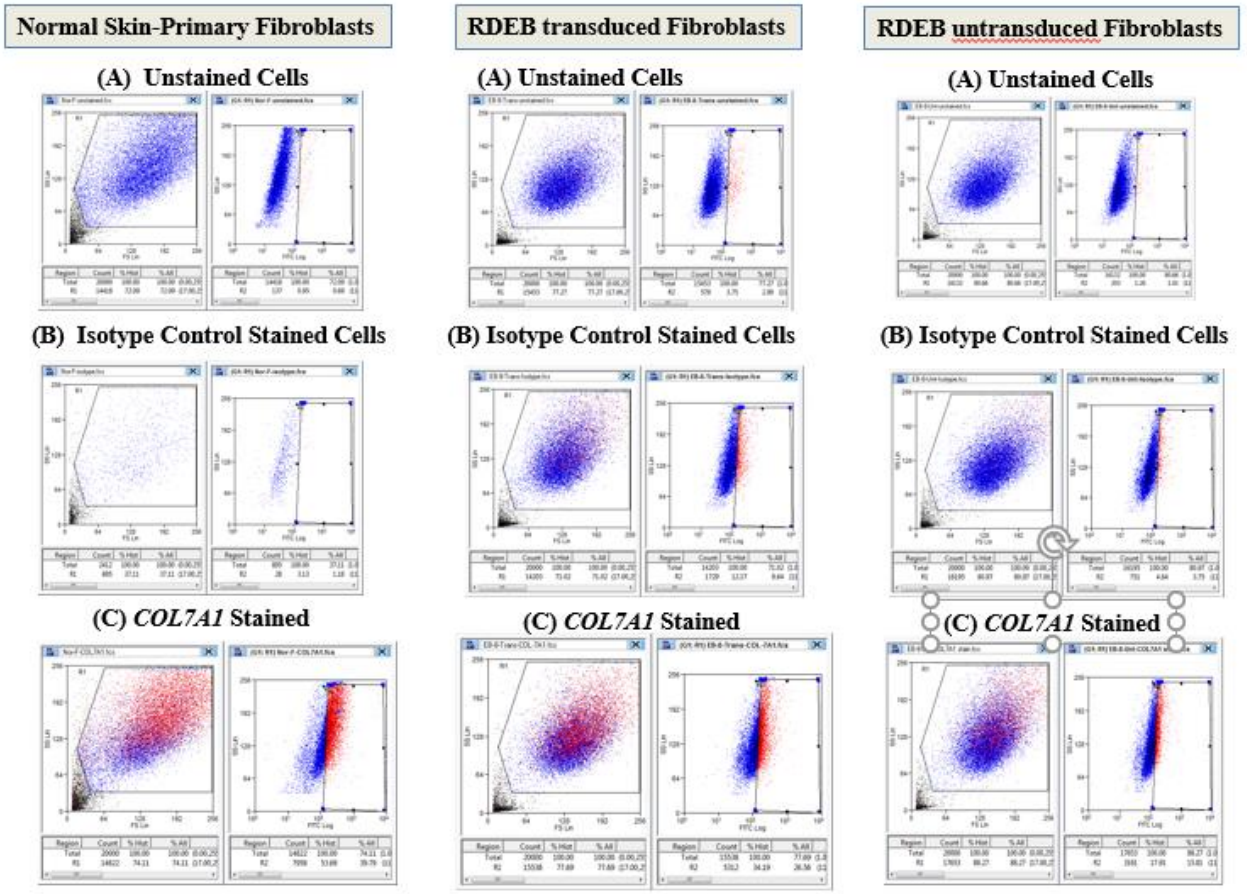


Figure 6-8 Flow cytometry analysis of C7 expression in pCCL-PGK COL7A1 fibroblasts compared to untransduced REB fibroblasts and wild type normal control fibroblasts. Gating strategy to gate fibroblasts on their FSC and SCC profile and then sequentially applied to C7+ population stained with Alexa Fluor 488 flurochrome correlating to FITC. Dot plot of positive C7 populations (red dots) in un transduced or transduced RDEB fibroblasts and wild type fibroblasts at Day 3 post transduction.

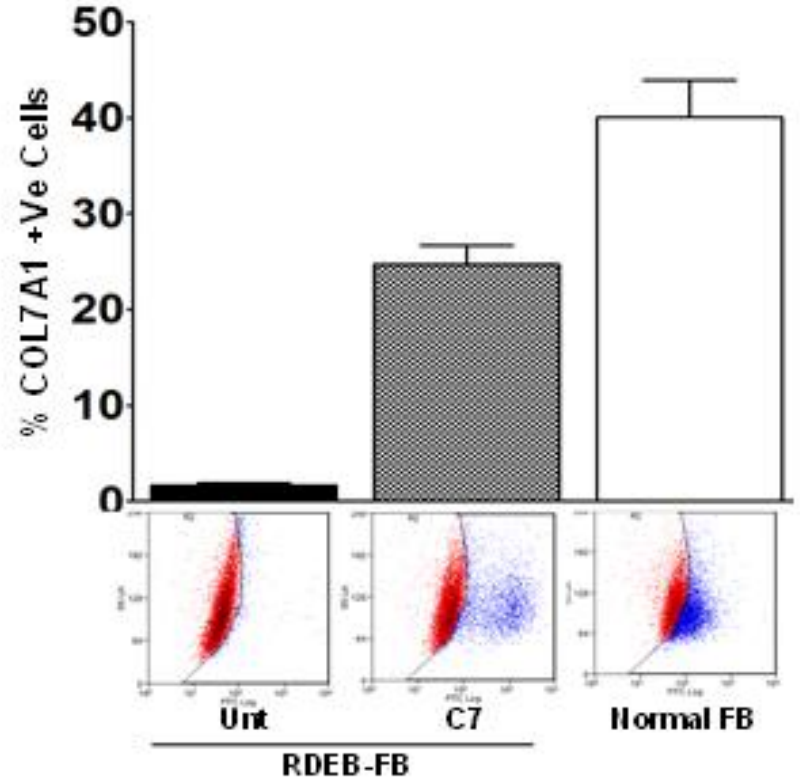


Figure 6-9 C7 Expression demonstrated using FACS analysis in gene corrected fibroblasts (RDEB-FB C7) compared to untransduced RDEB-FB and normal wild type fibroblasts from a healthy control

RDEB fibroblasts transduced with LV-COL7 vector were stained for intracellular C7 expression 2 weeks post viral exposure. Untransduced (UT) RDEB and wild-type (Normal) fibroblasts representing experimental controls were run in parallel. Flow cytometry shows positive C7 expression in LV-COL7 transduced RDEB and WT fibroblasts, but not in the UT cells. Isotype staining confirmed specificity of secondary antibody. Triplicate values of flow cytometry data were plotted against each other. Error bars represent SD amongst triplicates.

6.3.4 Functional *in vitro* assessment of transduced fibroblasts

The absence of C7 at the DEJ has been associated with altered keratinocyte migration and invasion *in vitro*. C7 corrected RDEB cells show functional restoration in migration assays as gene corrected RDEB cells efficiently closed the wound as compared to null C7 RDEB cells. There have been

contradictory reports concerning the effect on *in vitro* wound closure of keratinocytes in the absence of C7 expression. Originally described by Chen et al. (Chen et al., 2000b, Chen et al., 2002a), RDEB keratinocytes (KCs) appear to migrate in an inverse fashion to our findings with wild type (WT) KCs having a lower migration index value. This observation, however, might be explained by different protocols, with Chen *et al.* (2000) coating the cell culture surface with type I collagen prior to seeding and performing a colloidal gold migration assay. My assay, on the other hand, involves fibroblasts in untreated tissue culture dishes cultured to confluency before performing the migration assay, thereby allowing for the secretion and deposition of extracellular matrix proteins (collagens, laminin, fibronectin). Nystrom et al. (2013) demonstrated a significant reduction in migration speed of *COL7A1*-hypomorphic mouse keratinocytes, expressing 10% of normal C7 levels, when compared to wild type KCs, and similarly myofibroblast migration and maturation in granulation tissue was delayed (Nystrom et al., 2013). This finding was proposed to have been mediated by a direct interaction of C7 with the laminin-332/integrin α6β4 complex that is known to influence directed/directional migration of keratinocytes (Pullar et al., 2006, Sehgal et al., 2006, Tsuruta et al., 2011). Hence the reduced migration speed in the monoallelic and biallelic *COL7A1* keratinocyte knockouts observed, can be most likely explained by aberrant laminin-332 organisation altering α6β4 signalling caused by the knockout and lack of C7 protein deposition. A similar pattern was observed in C7-deficient fibroblast cells, showing that C7 expression has a keratinocyte independent effect on dermal fibroblast migration. Based on these functional studies RDEB fibroblasts

(MJ-05) having undergone transduction with pCCL-PGK-*COL7A1* LV were assessed for their ability to occupy an artificially created migration zone using methods previously described. Migration data from primary RDEB fibroblasts taken from a patient with partial C7 expression revealed increased wound closure speed of Col7a1(+) RDEB transduced fibroblasts compared with untrasnduced fibroblasts after 30 hours.

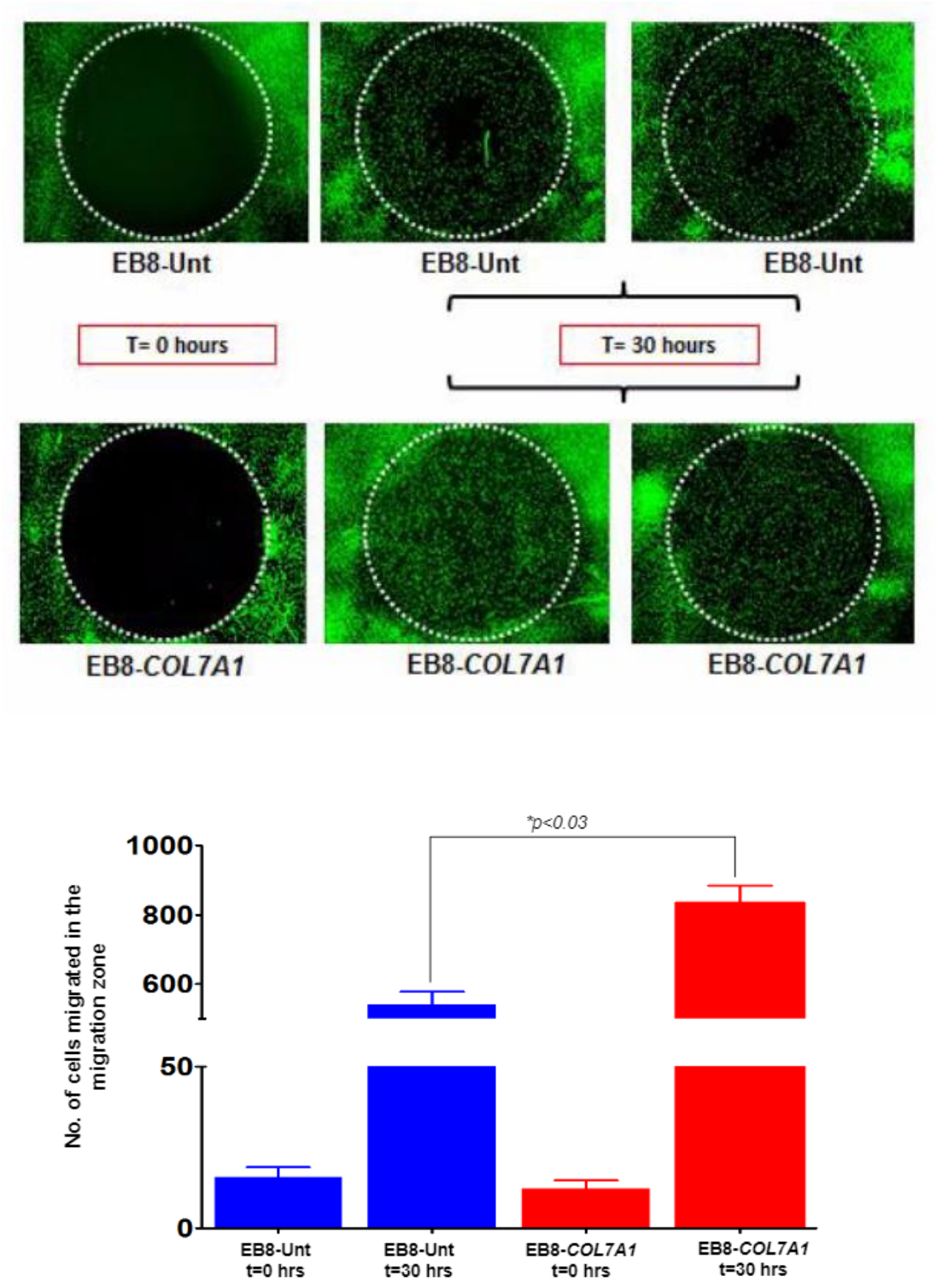


Figure 6-10 Migration of primary RDEB fibroblasts is increased following transduction with pCCL-PGK-COL7 LV vector.

Primary RDEB patient (EB8) fibroblasts either untransduced or following transduction were assessed for cell migration. The migration zone was imaged at t =0 and t=30. Increased migration was noted in transduced fibroblasts compared with untransduced ($p<0.03$).

6.3.5 Isolation and expansion of sufficient number of gene corrected fibroblasts

My aim was to ensure that > 1 x 10^6 fibroblasts would be generated according to GMP protocol. Skin biopsies taken from patients with RDEB were sampled to assess optimum biopsy size. 3mm, 4mm and 6mm punch biopsies were taken from RDEB patients in order to harvest fibroblasts. The optimum size punch biopsy was 6mm, as this sample was able to generate 5.7 x 10^6 cells following vector transduction and three passages (4-5 weeks) as well as 7 x 10^6 untransduced cells. In order to reduce the amount of time needed to expand sufficient cell numbers, I also opted to use an alternative method to the standard explant technique. Based on normal control skin biopsies, sufficient fibroblasts would only be cultured within 6- weeks. The use of collagenase D was shown to reduce the time to first passage compared with the explant technique, therefore minimising the time required within the GMP Cellular Therapies laboratory. The method involves digestion of the skin biopsy for two hours after soaking off the transport media with sterile PBS and peeling off the epidermis. Dissociated fibroblasts and the remaining dermal material are then seeded in a T25 flask

Table 6.2 Table showing the passage of primary RDEB fibroblasts from varying punch biopsy sizes.

Cells	lab label	sample size	digestion date (D0)	Passage I		Passage II		Passage III	
				date	cell no	date	cell no	date	cell no
RDEB	P28-FB	4mm	27-02-2013	D9	3x10^4	D21	1.5x10^5	D28	1.6x10^6
RDEB	P28-FB	6mm	27-02-2013	D10	2.3x10^5	D21	2.2x10^6	D28	7x10^6
RDEB	EB8-FB	5mm	21-03-2013	D10	1.2 x 10^5	D21	1.7 x 10^6	D28	4 x 10^6

6.4 Discussion

This data indicates that *ex-vivo* gene transfer to a modest fraction of fibroblasts using this vector results in high levels of C7 expression. The vector supports supra-normal levels of protein expression in transduced cells, as indicated by the high intensities of C7 detected by western blot and flow cytometry. Transduction efficiency at a cellular level is usually a reflection of the titre of the viral vector. In this study, the titre was limited by the large size of transgene insert, *COL7A1*. Reduction was attempted by using a codon optimised sequence (as described) however only a titre of 1×10^7 was achievable. The usual titre achieved in other gene therapy clinical trials using LV titres is generally between 1×10^8 and 1×10^9. Lentiviral vector titre is a critical factor influencing transduction efficiency. In CD34+ cells transduction frequency is related directly to vector concentration and not to multiplicity of infection (Haas et al., 2000). However, the necessity for higher levels of transduction efficiency in this clinical scenario are debateable. It is known that carriers of a single *COL7A1* mutations only producing 50% of wild type C7 levels are phenotypically normal, and therefore the level of protein required to maintain an intact epidermis is between 0% and 50%. Fritsch *et al* (2008) have shown that only very small increases in C7, up to 35% of normal levels, can significantly stabilise the skin against shearing forces and ameliorate the phenotype of a hypomorphic mouse model of RDEB (Fritsch et al., 2008). A level of gene correction up to 20% of baseline as demonstrated in this pre-clinical study would still feasibly still ameliorate the RDEB phenotype.

Further optimisation of levels of gene correction could be achieved by sorting the cultured fibroblasts post transduction, selecting only the C7(+) cells for propagation and use in a clinical trial. This could be theoretically facilitated by the use of a cell surface marker, such as CD34, vimentin, collagen I, fibroblast surface protein or α smooth muscle actin to enable sorting using a magnetic labelling system, however, in order to apply to a phase I study further validation and safety studies repeated using this surface marker are necessary.

Titeux *et al* (2010) have shown that rearrangements occur at both a protein and genomic level following transduction of both keratinocyte and fibroblast populations using a SIN retroviral vector with an EF1α promoter (Titeux et al., 2010). The clinical significance of these genomic rearrangements is unknown. Retroviruses are known to have a relatively high mutation rate contributing to their evolutionary success. Significant levels of genomic rearrangements are known to occur within both gamma-retroviral and lentiviral viruses (Jetzt et al., 2000, De Angioletti et al., 2002). The frequency of the mutation rate in the coding region of eukaryotic cells by retroviral vectors has been examined in the human glucose-6-phosphate dehydrogenase gene transferred by Moloney murine leukemia virus based vectors into murine bone marrow cells. Abnormal hG6PD variants were estimated to occur at a relatively low rate of 1.4×10^{-5} per base pair per replication cycle (De Angioletti et al., 2002). Genomic rearrangements have been shown to occur in 3 out of 50 keratinocyte clones transduced using the pCCL-PGK-COL7 vector with deletions occurring within genomic DNA leading to truncated C7 protein bands (F Syed, co-worker at Institute of Child Health,

unpublished work). Following single cell cloning the majority of transduced clones expressed full length C7 but ~6% had truncated versions of the protein. Further sanger sequencing identified 3 deletions in these clones between 5200bp and 7900bp likely to have arisen during reverse transcription due to repeat sequences within the C7 gene. The clinical relevance of a small percentage of truncated protein is likely to be negligible. Dominant-negative interference in protein expression is known to occur following heterozygous glycine substitutions (Fritsch et al., 2009) or exon skipping (Toyonaga et al., 2014) of *COL7A1*. Mutant truncated forms of C7 due to in-frame deletions in *COL7A1* are present physiologically in patients with RDEB with milder phenotypes with no evidence of protein degradation or deleterious clinical consequences (Terracina et al., 1998, McGrath et al., 1999). It should therefore be anticipated that the small percentage of gene corrected fibroblasts synthesising truncated forms of C7 should not impact the formation of anchoring fibrils or lead to worsening disease, although the efficiency of therapy would be enhanced with a reduction in truncated forms.

Further concerns from a regulator's perspective may be related to the risks of over-expression of the target protein. Pre-clinical studies using retroviral driven expression of full length C7 in cultured keratinocytes greatly exceeds those seen in wild type control cells (Gache et al., 2004a). Studies assessing the impact of C7 over-expression are mainly concerned with the risks of SCC development. *In vitro* studies have shown that manipulation of C7 yields varying results depending on the cellular context and approach used (Martins et al., 2009, Ortiz-Urda et al., 2005). Many RDEB tumours do not express C7 and patients can develop SCCs regardless of baseline C7 expression levels

(Pourreyron et al., 2007). Over-expression of C7 in dermal fibroblasts has been shown to reduce tumour invasion and tumour formation in RDEB keratinocytes (Ng et al., 2012). Studies attempting to over-express C7 in cultured RDEB keratinocytes (up to 35 fold) using recombinant *COL7A1* cDNA in a retroviral vector show that supra-physiological levels of C7 are associated with increased invasion and migration of keratinocytes in RDEB SCC keratinocytes, associated with an activation of the phosphoniositide 3 kinase pathway (Pourreyron et al., 2014). However, the results of this study have not shown significant levels of fibroblast over-expression in gene corrected cells using this pCCL-PGK-COL7 LV vector. Assessment of cellular migration in *COL7A1*(+) RDEB cells did not exceed those of RDEB *COL7A1*(-) cells.

Production and release of a clinical batch of LV-*COL7A1* vector has been performed and demonstrated engineering of human RDEB fibroblasts under GMP conditions. These data have been used to support the application for MHRA approval for a phase 1 study.

6.5 Summary

This chapter summarises the preclinical data generated related to the transduction efficiency of the LV-COL7A1 vector in primary RDEB fibroblasts.

(1) Transduction efficiency assessed using both qPCR and FACS analysis showed up to ~20% transduction efficiency reducing down to 9% at 3-4 weeks post transduction
(2) Increased levels of C7 were demonstrated using both IF and western blot

(3) Functional assays confirmed increased migration in transduced cell types
(4) Cell toxicity was demonstrated at higher MOI (>20)

This data was included as part of the Investigators Brochure required for the MHRA application for the LENTICOL-F phase I clinical trial.

Chapter 7

Protocol design for a phase I study of gene corrected intradermal fibroblasts in RDEB

7.1 Introduction

One of the main ambitions at the start of my research period was to design and launch a phase 1 study of autologous intradermal gene corrected fibroblasts in RDEB. This was following discussion and collaboration with Dr Waseem Qasim and Professor Adrian Thrasher at the Institute of Child Health. In anticipation of the pre-clinical safety data, one of my roles was to develop the clinical trial protocol and the practical development of the trial, running in parallel to the pre-clinical work. This would ensure a speedy application to both the ethics committee as well as the MHRA in order to take this forward to a human clinical trial. Applications for gene therapy clinical trials can be extremely protracted and time consuming and therefore this work running concurrently would rapidly accelerate the launch of the clinical trial.

If successful, further systemic therapies would be planned using the same vector platform and could lead to new therapeutic avenues for RDEB and other skin diseases.

This chapter details my work and initial protocol development for the phase 1 study. My experience working collaboratively on developing phase 1 trials of intravenous mesenchymal stromal cells for children with RDEB (EBSTEM) as well as facilitating with a phase I trial of a gene corrected

epidermal graft for adults and children with Netherton syndrome aided to my expertise in this area.

7.2 Study design

The main aim was to assess whether intradermally administered self-inactivating (SIN) lentiviral vector (LV)-mediated *COL7A1* gene-corrected autologous fibroblasts are safe and to evaluate their potential efficacy in adults with RDEB. This would be a Phase 1, non-randomised, open-label, single-centre, proof-of-concept study. The ideal sample size would be 10 adult patients with RDEB, with a minimum of 5 patients. Following advice from our statistician, even if no subjects report side effects (0%), a sample size of 10 would give a 95% confidence interval of 0% to 31% (exact CI from Stata®. A sample of 10 could still provide reassurance that no more that 3 in 10 subjects should experience a given side effect. Recruitment in rare disorders can be extremely challenging, particularly with recruitment of adult patients with RDEB whom have complex medical needs and often more significant social limitations affecting the ability to recruit to phase I clinical trials as compared with the paediatric population.

As the purpose of a phase I study is the demonstration of short to medium term safety at both a local and systemic basis, an active comparator such as allogeneic fibroblasts would not be necessary. However, ideally a control of a presumed non-active control comparator would be used and therefore the study would also assess non-gene corrected autologous fibroblasts. Comparing two cell populations at three time points would require six

biopsies per patient, excluding the larger biopsy required for harvesting fibroblasts.

7.3 Trial protocol

I designed the basis for the LENTICOL study which underwent at least 3 version changes during the research period. The design of the trial protocol was based on previous work performed by our group using allogeneic fibroblasts. Wong *et al* (2007) performed the first study of allogeneic fibroblasts injected into RDEB intact skin. This study protocol utilised three cell types (patients' own autologous fibroblasts, allogeneic fibroblasts derived from a parent and allogeneic fibroblasts from an unrelated donor). Biopsies were carried out at 2 weeks and 3 months. All five subjects demonstrated an increase in C7 immunolabelling at the DEJ at 2 weeks after allogeneic fibroblast injection, compared with baseline.

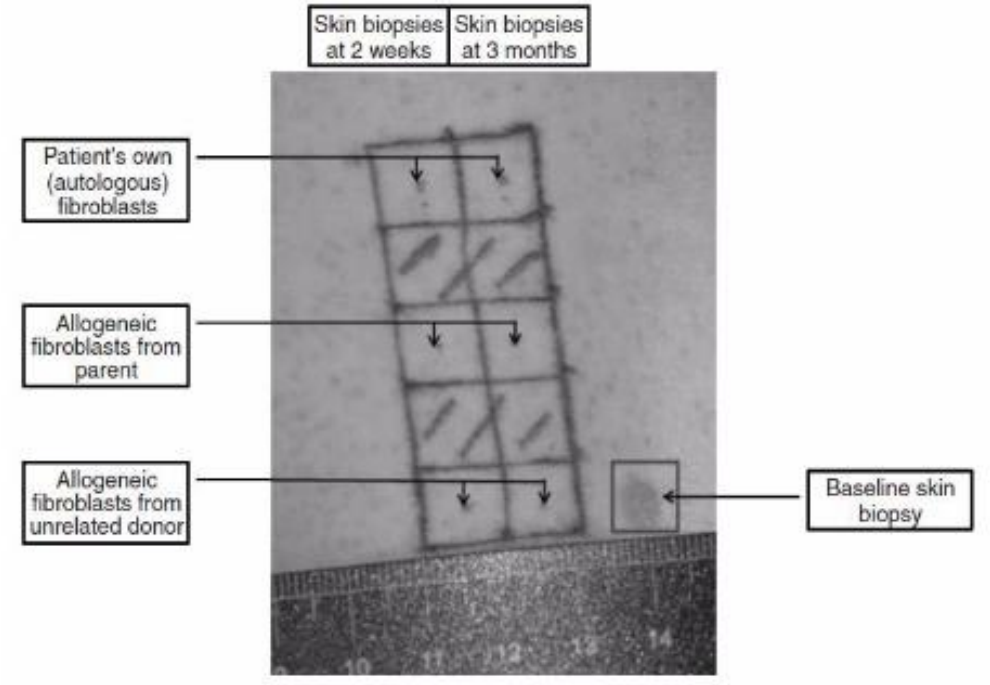


Figure 7-1 Fibroblast injection site map and biopsy time points performed by Wong et al 2007.

Grids measuring 1cm^2 were drawn onto intact, non-blistered skin of each subject. The baseline skin biopsy used to generate patients own autologous cells and to determine baseline parameters is shown. Six injections were administered as shown and biopsies were then taken at either at 2 weeks or 3 months.

The study design for LENTICOL-F is illustrated in Figure 7.2 and will utilise two cell types only : autologous pCCL-PGK-COL7 gene corrected fibroblasts (IMP) and autologous untransduced fibroblasts (control). Each study participant will receive three intradermal injections of each cell type on Day 0 only. Each injection (both IMP and control) containing 0.8–1.2x10 cells suspended in 0.25ml of 0.9% saline, will be administered intradermally into separate 1cm areas of intact skin as shown on the grid map of injection sites (see Figure 7.3).

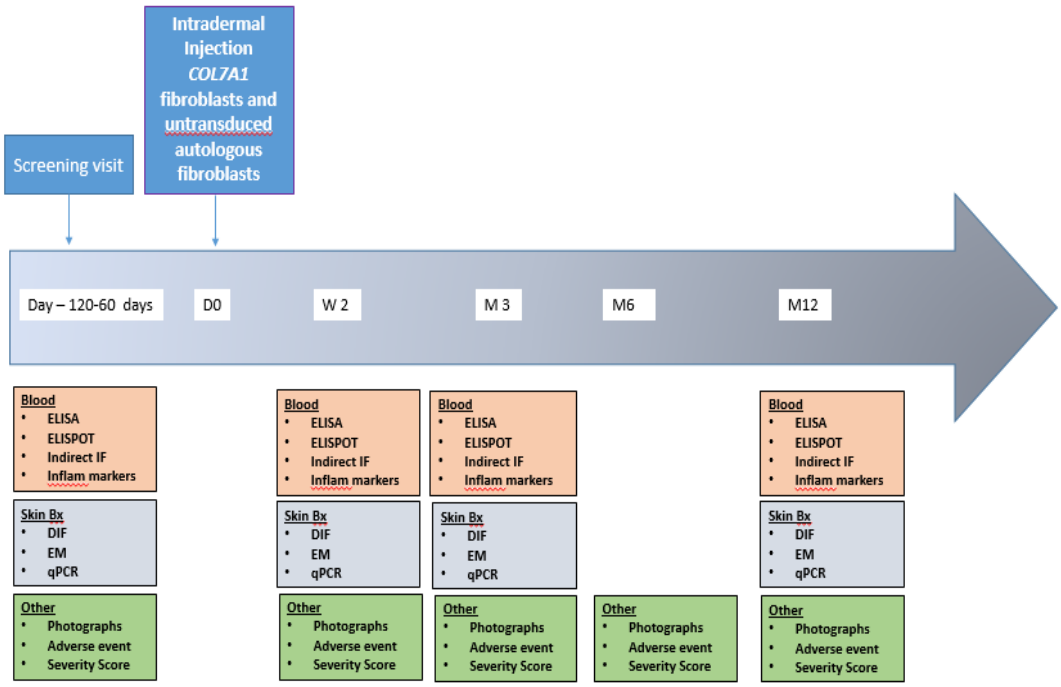


Figure 7-2 Flowchart showing the planned visits and procedures for the first 12 months of the clinical trial

D0= Day 0, W2 = Week 2, M3 = Month 3, M6= Month 6, M12 = Month 12, Inflam markers = Bloods including full blood count, urea and electrolytes, liver function tests, bone profile, lactate dehydrogenase, erythrocyte sedimentation rate and C-reactive protein, DIF = Direct Immunofluorescence, EM = Electron Microscopy, qPCR = transduction efficiency assessment of vector copy number using qPCR.

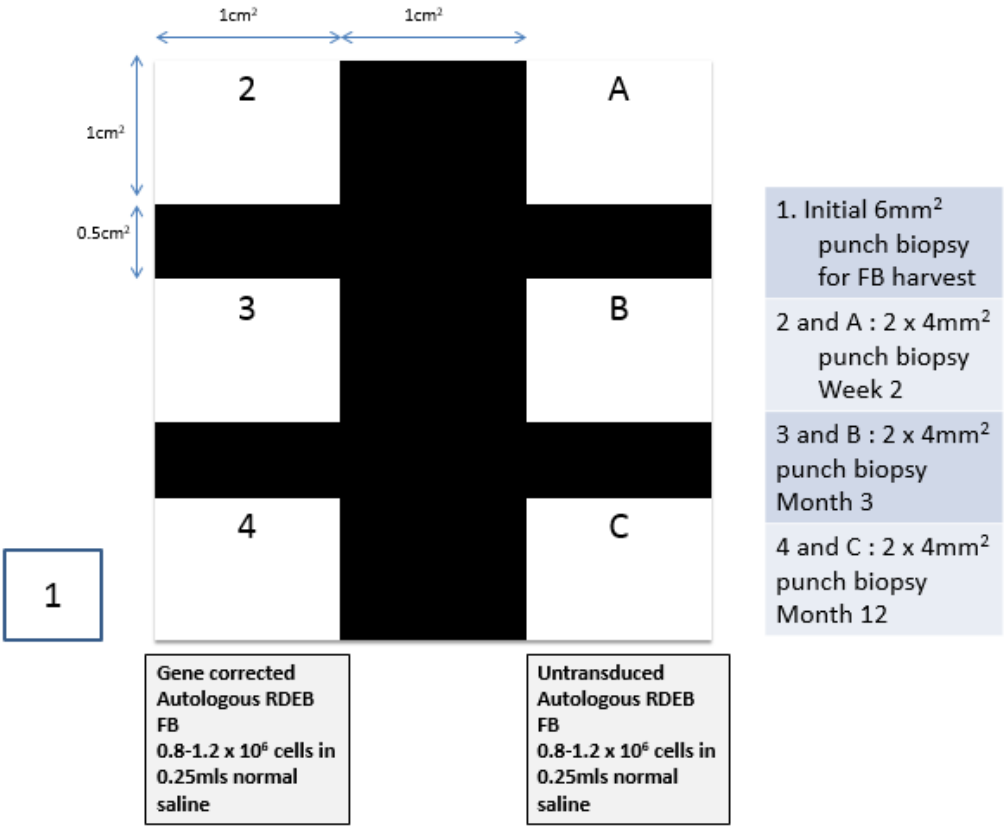


Figure 7-3 Planned injection site map on intact skin

The shaded blocks show areas that will not be injected or subsequently biopsied. In order to ensure the grid is replicated on each visit a "tattoo" will be placed using an indelible marker pen in order to orientate the transparency with the gridlines. Each biopsy site will need to have 2 x 4mm punch biopsies taken to ensure sufficient material for IMF, EM and qPCR. All patients will have previous baseline values performed as part of screening.

Subsequent biopsies would be sampled at week 2, month 3 and month 12 post administration and compared with baseline biopsy data. From the injection site skin biopsies: (1) presence of C7 protein by direct immunofluorescence (DIF); (2) presence of anchoring fibrils at the dermal-epidermal junction by transmission electron microscopy (TEM) (3) transduction efficiency using vector copy number assessed via qPCR. From the blood/serum: full blood count (FBC), urea and electrolytes (U&Es), liver function tests (LFTs), bone profile, lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) to assess possible systemic adverse effects and

a change in generalised inflammation at W2, W4, M3, M6, M9 and M12 compared to baseline. Serum would also be used to assess ELISA and IIF in order to detect any potential development of EB acquisita as a potential adverse effect of introducing new C7 expression from the gene-modified autologous fibroblasts. ELISPOT assay for detection of B- and T-cell responses to the full length C7 will be performed at screening, W4, M3, M6, M12, M24 and M36.

Local adverse effects should be assessed by clinical reviews, medical photographs of the biopsy site performed at each visit as well the review of diary cards completed by each patient during the study. Possible systemic effects will be assessed by physical examination and vital signs assessment performed by the clinician at each visit, review of diary cards and review of blood analysis indicating possible systemic immune response to the IMP.

Patients will be followed up with study interventions for a 12 month period and then followed up quarterly for the second year and semi-annually for the third year as part of routine clinical care. Previous studies approved by GTAC in the past 5 years have usually requested a 36 month follow-up period in order to ensure safety data is accurately assessed with a particular concern regarding possible genotoxicity. As the number of gene therapy clinical trials continues to increase the likely necessity for prolonged assessment periods may be reduced.

7.4 Inclusion and Exclusion criteria

Participants of either sex (over the age of 16) would be invited to participate if they had a diagnosis of RDEB, based on clinical and molecular findings. Patients with a history of a previous SCC would be excluded due to the risk of harvesting dysplastic epithelium. Criteria are listed in the table below.

Inclusion criteria

- Subjects with a clinical diagnosis of RDEB as confirmed by clinical characteristics and mutation in the COL7A1 gene
- Subjects who have partial or complete absence of C7 on DIF
- Subjects whom have at least $15cm^2$ of intact skin on the trunk and/or the extremities
- Subjects who are aged ≥ 16
- Subjects who have voluntarily signed and dated an informed consent form prior to the first study intervention
- Subjects, who are, in the opinion of the investigator, able to understand the study, cooperate with the study procedures, and are willing to be available for all the required follow-up visits

Exclusion criteria

- Subjects who have received immunotherapy/chemotherapy within 90 days of enrolment into this study
- Subjects with a known allergy to any of the constituents of the product
- Subjects with known or suspected malignancy, including cutaneous malignancy
- Subjects with intolerance or allergy to additional study associated drugs/therapies (e.g. local anaesthetics, etc.)
- Subjects who have taken systemic antibiotics within 7 days
- Subjects taking immunosuppressive therapy including systemic steroids (e.g. oral prednisolone > 40 mg for more than 1 week) within the 30 days of the first treatment or planning immunosuppressive

therapy at any time during the study. Intranasal/inhaled steroids are acceptable

- Subjects who have taken any other investigational product within 90 days prior to screening or planned use of any other investigational product during the study period
- Subjects who are pregnant, planning pregnancy and women of childbearing potential who are not abstinent or practicing an acceptable means of contraception, as determined by the investigator, for the duration of the treatment phase
- Subjects with active infection requiring intravenous antibiotics at time of recruitment or administration of the IMP
- Subjects with positive results for blood-borne pathogens (under 2006/17/EC) : HIV, Hepatitis B surface antigen (HepBsAg), Hepatitis B core antibodies (HepBcAb), Hepatitis C IgG (HepCIgG), human T-lymphotropic virus (HTLV) and *Treponema pallidum* serology for Syphilis
- Subjects with a known history of poor adherence/compliance with medical treatment or follow-up
- Subjects who are unable to understand the aims, objectives and follow-up treatment
- Subjects with known alcohol or narcotic drug dependence.
- Subjects who have who have an immune response to C7 as evidenced by both
    - positive serum antibodies to C7 confirmed by ELISA or ELISPOT **and**
    - positive IIF with binding to the base of salt split skin.

7.5 Manufacture of the IMP

7.5.1 Isolation of primary fibroblasts from skin biopsy using digestion method

A 6 mm punch biopsy is taken from a limb. The biopsy is immediately placed in transfer medium (DMEM/10% FCS) in a sterile bottle, kept at 4^0C and transported to the laboratory within 12 hours. After the skin biopsy is delivered to the laboratory, it is immediately digested with neutral protease

for 30mins to remove the epidermis, followed by digestion with collagenase NB6 for 2 hours to isolate fibroblasts incubated at 37 degrees. Following digestion, the solution is centrifuged at 200G for 10 minutes and the supernatant removed carefully and resuspended in 3 mls cell culture media (Dulbecco Modified Eagle Media supplemented with 10% v/v FCS) and plated into two T75 flasks.

7.5.2 Subculture and storage of primary fibroblasts

The first passage of cells will take place approximately 14 days after initial seeding. Each flask is washed with PBS without calcium or magnesium and then supplemented with 5 mls of 0.05% Trypsin/EDTA and incubated at 37^{C} until the cells are fully detached. Equal volumes of DMEM/FCS cell culture media are added and the cell suspension is transferred from the flask to a centrifuge tube. The cell pellet is replated in a new flask for continuing culture and transduction.

7.5.3 Transduction of primary fibroblasts

After no more than 4 cell passages, a total of $10x10^{6}$ fibroblasts 2ill be subcultured in two T25 flasks. One flask is used for generating the patient's gene-modified fibroblasts and another flask is used to generate non-modified fibroblasts. Twenty four – forty-eight hours after seeding, cells will undergo one round of transduction with pCCL-PGK-COL7A1 vector. The following day, the media is replaced with fresh culture medium. Both transduced and untransduced cells are then continuously cultured until reach 80% confluence (approximately 5-7 x 10^{6} cells) which takes approximately 1-2 weeks. Cells are trypsinised and counted 2 to 3 weeks post-transduction. 1x10 cells are

taken from both transduced and untransduced cells for characterising gene modified fibroblasts. The assays include pro-viral copy number and C7 expression in cells.

7.5.4 Cryopreservation of IMP

Harvested cells are washed and resuspended in freezing media containing FBS/10% DMSO. 3 vials of transduced cells and 3 vials of untransduced cells are frozen with 2x10 cells/cryo-vial and stored in a controlled grade -80C freezer. 24 to 48 hours later, cells are transferred into the liquid nitrogen vessel linked to a temperature monitoring/alarm system.

7.5.5 Thawing and dispatch of fibroblasts

Frozen transduced and untransduced cells are thawed and washed with PBS. Cells are resuspended in PBS and counted. After counting, cells are re-pelleted, and resuspended in saline with approximately concentration of 3.2–4.8 $x10^6$ live cells/ml 0.25 ml cell suspensions containing 0.8 -1.2 x 10^6 cell count live cells are packed into each 1ml syringe capped with luer-screw. Three syringes are packed with untransduced cells and three syringes are packed with transduced cells. All syringes are clear labelled and then packed into a transfer container for transport at ambient temperature.

7.6 Discussion and update

Based on my work with the GENEGRAFT project and protocols, there were a number of practical limitations and considerations which have delayed the regulatory applications and approvals, such that almost 6 years after the project was started we are yet to graft the first patient (November 2016). Running alongside my goal was to work on the LENTICOL-F project in order

to ensure a gene therapy protocol using gene corrected autologous fibroblasts administered intradermally could be delivered in the UK in a timely way. At the start of my research period, I had already been in discussion with Waseem Qasim and Adrian Thrasher at the Institute of Child Health regarding the project. Their group had viral vector platform that had been approved previously by the MHRA for another condition (Netherton syndrome) and already had a grant approval for the pre-clinical work. My goal was to design the clinical trial protocol in anticipation of the safety data results in order to take forward into a clinical trial. Based on our experiences in the preparation for gene therapy clinical trials, it was clear waiting for the preclinical data before preparing the trial application would lead to significant delays. My work was critical for accelerating the launch of the phase 1 trial ensuring sufficient data for the MHRA application including the documents required for the trial protocol and Investigator's Brochure (See supplementary material). I was able to take lessons learnt from my experience with the GENERGRAFT trial as well as the EBSTEM trial and work on the Netherton gene therapy trial to fast track this clinical trial, anticipating potential problems and therefore avoiding unnecessary delays. My period of research was completed in January 2015, but my work ensured that the MHRA application was completed on the 30th March 2015 (EudraCT 2014-00484-19), GTAC meeting was on the 20th May 2015 and the first patient was screened in August 2015.

7.7 Summary

This chapter summarises the my work on the development of the LENTICOL phase 1 clinical trial in terms of early protocol design including inclusion and

exclusion criteria, primary endpoints and trial design. In addition,my work included completing the Investigator Brochure detailing the specific IMP of autologous RDEB gene corrected fibroblasts. These documents facilitated the application for the trial to both GTAC and MHRA.

Chapter 8

Future perspectives

8.1 Short term goals

The pre-clinical data in Chapter 6 and clinical trial design in Chapter 7 have formed the basis for the successful application of the phase 1 study of autologous LV-COL7A1 corrected fibroblasts injected intradermally into adult patients with RDEB, LENTICOL-F (NCT0493816). Three patients have now been recruited and administered the IMP, with data regarding the subsequent follow-up biopsies still pending. Skin biopsy analyses are ongoing. Following on from this trial, the next logical step would be to design a clinical trial of a systemic therapy (intravenous or intrarterial administration) using either fibroblasts or MSCs corrected using the same vector platform. The benefits of systemic therapy would be the targeting of multiple chronic wounds simultaneously as well as the possible benefits in terms of mucosal involvement that would be otherwise impossible to achieve with grafting or intradermal therapies alone. Intravenous administration of both allogeneic fibroblasts and gene corrected fibroblasts have been shown to home to the skin and deliver C7 to the BMZ in murine skin (Woodley et al., 2007). Interestingly only the corrected fibroblasts over-expressing C7, but not

uncorrected fibroblasts, led to increased rates of wound healing. A small number of fibroblasts detected in foci of lung tissue in these mice raised a possible safety issue (although there were no pathophysiological sequelae in the murine models), hence a possible consideration for intrarterial administration of therapies. However, data recently published showing the safety of intravenous MSCs in both adults and children with RDEB (Petrof et al., 2015, El-Darouti et al., 2016) is supportive of this modality for administration, and considering the patient population would be less invasive and easier to perform. Furthermore, the assumption would be that in immunocompetent patients, cells migrated to unintended sites would be rapidly cleared (Woodley et al., 2007). Data from the currently active phase I study of intravenous allogeneic MSCs in adults with RDEB (ADSTEM NCT02323789) will be critical in determining which cell type should be the next target for gene correction and systemic administration, as it is currently unclear which stem cell population would be able to show the greatest clinical benefit in RDEB. ADSTEM is a phase I clinical trial based at GSTT/KCL recruiting up to 10 adult patients with RDEB and administering 3 infusions of allogeneic MSCs over 14 days. MSCs in particular have been shown to migrate to wounded sites and contribute to tissue regeneration while also confer immunomodulatory properties (Fathke et al., 2004b, Wang et al., 2014). Currently systemic delivery of MSCs are being tested in clinical trials in a wide variety of systemic inflammatory and autoimmune conditions that affect the skin, such as systemic lupus erythematosus and graft versus host disease, and therefore there is a significant amount of safety data using this as an ATMP. In fact recently Kühl *et al*. found that human MSCs express

C7 *in vitro* at approximately the same level as fibroblasts (Kuhl et al., 2015); and that in addition to secreting C7, they are able to mobilize myofibroblasts, ameliorate skin inflammation, and increase skin stability. My hope would be to develop a further study, LENTICOL-M, using gene corrected MSCs, in patients with severe RDEB and chronic wounds.

Other considerations for the use of gene corrected fibroblasts should be in the development of skin bio-equivalents and grafting. RDEB patients typically develop multiple sites of SCCs requiring surgical excision. Diseased fibroblasts such as those in RDEB have been shown to promote tumour progression, growth and metastasis by facilitating angiogenesis at the site highlighting the need for their removal (Flavell et al., 2008). Fibroblasts have also been shown to have a role in cancer at all stages including progression (Ronnov-Jessen et al., 1996), growth (Dvorak, 1986) and metastasis (Schedin and Elias, 2004). Therefore corrected fibroblasts introduced at graft site would compete with diseased fibroblasts and could theoretically benefit areas at high risk of further dysplasia. A clinically bioengineered scaffold similar to Apligraf® but containing autologous gene corrected fibroblasts overlayed with an autologous epidermal sheet could therefore be used to "fill" the wound following excision of SCCs. During my research period two of the nine recruited patients developed new SCCs requiring grafting, and therefore this option could be of significant benefit for them.

Further goals in the field would be for the implementation of the GENEGRAFT study, and administration of the first gene-corrected skin equivalent graft for a UK patient with RDEB. The EU project has faced

multiple challenges during this time period since the initial kick off meeting in May 2010. There have been issues regarding the need for GMP facilities required for manufacture of the stem cell therapies. Key components of GMP accreditation include: (a) performance of best-practice standard operating procedures when conducting research; (b) research staff being trained to a GMP standard; and (c) the building of an accredited GMP clean room where air pressure, temperature and sterility are monitored (Stephens et al., 2011). Up until 2012 GLP practices were used and the transition to GMP facilities would require complete redesign and refurbishment likely to take at least 5 years to set up. This issue as well as inordinate volumes of pre-clinical safety data required to satisfy the EU regulatory bodies as well as conflicts within the multinational group led to changes to the site of manufacture of the IMP from Paris (INSERM), to Modena (Centre for Regenerative Medicine, UNIMORE) and finally now to Madrid (CIEMAT, Centro de Investigaciones Energeticas , Medioambientales y Technologicas). The need to ensure that the skin equivalent graft would be cultured and transduced in a GMP approved laboratory halted the initial plans for the IMP manufacture to be performed in Paris. Each transition has required repeat GMP runs and development of SOPs relevant to each site. This has naturally led to significant delays in the delivery of this clinical trial, but I would still be hopeful for the data generated from Chapter 3 to form the basis for patient selection for the study and also to have helped developed SOPs for the patient pathway necessary for recruitment of patients from the UK. Regarding the applicability of gene corrected grafts for patients with RDEB, I do envisage them to have a role relevant for certain patients with localised disease,

particularly patients with RDEB-inversa with recalcitrant, chronic wounds. The Stanford group have recently published their data set for 4 patients (n=24 grafts) grafted with autologous epidermal sheets but in one individual they have demonstrated that multiple sites can be treated simultaneously (Siprashvili et al., 2016). C7 expression at the DEJ was demonstrated on the graft sites by immunofluorescence microscopy in 9 of 10 biopsy samples (90%) at 3 months, in 8 of 12 samples (66%) at 6 months, and in 5 of 12 samples (42%) at 12 months, including correct type VII collagen localization to anchoring fibrils. The decline in expression is different from the outcome described by Mavilio et al (2006) in their case report of a grafted patient with JEB, and may reflect a number of factors including the enrichment of stem cells in culture as well as the stem cells accrued from the harvest biopsy. The team have highlighted the importance of post-surgical procedures and variability according to graft site, as predicted by the GENEGRAFT project. Primary RDEB keratinocytes were transduced with the LZRSE-COL7A1 retroviral vector containing full-length *COL7A1* sequence and the long terminal repeat promoter with a mean of 70% efficiency and 0.8 pro-viral genome copies per cell. Interestingly that the wound beds were not induced and had been present for more than 5 years. The key important outcome was that no serious adverse safety events were reported, and particularly there was no evidence of a predisposition for the development of cutaneous SCCs, despite the inclusion of patients without a previous history of SCC. However, long term follow-up will be critical over the next 5 years to ensure a lack of oncogenic potential with this therapy. Immune response to the graft was not clinically relevant in all 4 of the treated patients, however all selected patients

expressed a non-functional truncated C7 protein containing the NC1 domain. Despite the lack of clinical sequalae, transient circulating antibodies were detected in 3 of the 4 patients using indirect immunofluorescence using patients' serum. In fact, one patient had a previous positive IIF result (only detected retrospectively) highlighting again that a higher than expected number of patients who express non-functional C7 protein may have pre-existing circulating antibodies but this may not necessarily lead to a negative clinical outcome.

Professor Johan Bauer's group in Austria have also demonstrated the applicability of grafting method in two patients with JEB (Murauer et al., 2015). A patient with JEB was selected in Salzburg, a skin biopsy sent to Modena for transduction and culture. Five skin sheets each measuring 5 × 7 cm, were grafted onto wounded areas on the patient's thighs. The one year followup has yet to be published but initial data presented at DEBRA International 2015 showed normal appearing skin at the graft site with no reported adverse effects. One patient with JEB and coincidental Staphylococcus Scalded Skin Syndrome was also treated with widespread grafting of genetically corrected epidermal sheets under a Specials license, with an extremely favourable outcome although the long-term data has not been presented (J.Bauer, British Association of Dermatology meeting 2016). I strongly believe that as long as the long term safety data is assured, this approach will have an important role to play in the management of chronic wounds in a subset of patients with RDEB.

8.2 The future for cell and gene therapies for RDEB

Gene therapy for RDEB is an expanding phase of development. Exciting advances in gene editing approaches, circumventing and minimising the theoretical risk of insertional mutagenesis. Furthermore, gene editing would also overcome the obstacle of delivering full length cDNA or potential complications arising from transgene over-expression that could contribute to C7 mediated fibrosis. As the molecular tools available are still in their infancy one would expect significant advances to be made through increased efficiency and improved safety profile.

The ultimate therapy for RDEB would be a combination of gene, cell, protein and drug therapies tailored according to the individual patient. Gene therapy clinical trials for RDEB have thus far focused on gene replacement using integrating viral vectors. Future directions will likely develop and build upon the current wave of phase 1 studies into phase II studies utilising cell types capable of systemic administration.

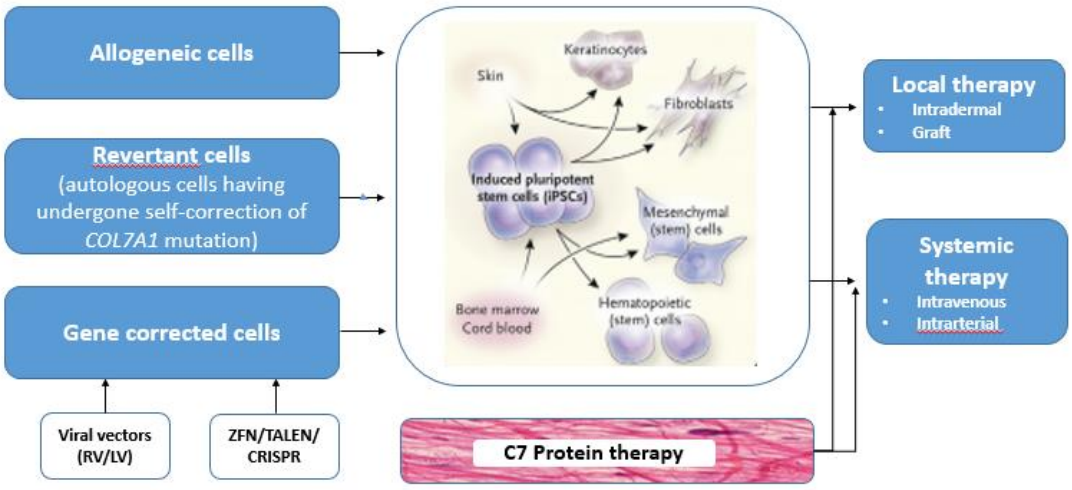


Figure 8-1 Combination therapy for RDEB

Adapted from Wagner and Tolar, New England Journal of Medicine (2015). RV = retroviral vector, LV = lentiviral vector, ZFN = Zinc finger nucleases, TALEN = Transcription activator-like effector nucleases, CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats

Bone marrow transplant is still a consideration for patients with severe RDEB and reduced intensity conditioning regimens are continuing, particularly in the USA. Geyer et al (2015) published further data on 2 children with RDEB treated with a reduced intensity regimen using busulfan, fludarabine and alemtuzamab followed by HLA matched unaffected sibling-donor unmanipulated bone marrow haematopoietic progenitor cells (HPC) (Geyer et al., 2015). Although the regimen is safer than the full intensity approach, the duration of effect on skin fragility and blistering appeared to be short term in these cases and *COL7A1* mRNA level levels were not sustained. Furthermore, the risks of giving allogeneic BM stem cells are still significant, particularly in a population with far higher premorbid state and with higher chances of developing severe, systemic infections. Recently there has one case of Toxic Epidermal Necrolysis (TEN) which developed following a

myeloablative allogeneic unrelated-donor 8/8 HLA-matched BMT and concurrent allogeneic marrow-derived MSC infusion (Boull et al., 2016). Given these risks, gene corrected autologous transplant of HPC should be the next approach.

Further studies using placental based cell therapies are also underway. In fact, umbilical cord blood (UCB) has several potential advantages over bone marrow (BM), including decreased collection risk to the donor compared with the harvesting of BM, decreased risk of infection transmission from donor to patient, a need for less stringent human leukocyte antigen (HLA)-matching requirements and an overall lower risk of graft-versus-host disease (GVHD) (Nevala-Plagemann et al., 2015). Cord blood banks are increasingly available and more than 20,000 CB transplants have been carried out worldwide. However, comparison of umbilical cord cells versus BM stem cells in individuals with RDEB has shown better skin engraftment with a BM-derived population (Tolar and Wagner, 2012).

An additional population of stromal stem cells that can be found in UCB are known as unrestricted somatic stem cells (USSCs) (Kogler et al., 2004). Recent, pre-clinical data have shown that unrestricted somatic stem cells can express C7 and accelerate wound healing, including improving the quality of wound healing, as evidenced by formation of new skin appendages (Liao et al., 2014).

8.3 Future gene editing strategies

Since commencing this period of research the field of gene editing has accelerated with exciting developments. In particular, the ease with which CRISPRCas9 and TALENs can be configured to recognize new genomic sequences has driven a revolution in genome editing. Subsequent to this thesis there have been clinical trials using gene editing platforms. Tebas *et al* (2014) treated 12 patients with HIV in an open label study using a single dose of CCR5 ZFN modified autologous CD4 T cells. The infusion of autologous CD4 T cells in which the CCR5 receptor had been rendered dysfunctional by ZFNs targeting *CCR5* were generally safe, with only one transfusion reaction noted.

In addition targeted nucleases can be combined with viral vectors, such as AAV vectors, to mediate genome editing in situ. In vivo genome editing has recently enabled the restoration of dystrophin gene expression (*DST*) and the rescue of muscle function in mouse models of Duchenne muscular dystrophy.

Despite these exciting advances there still are a number of hurdles to overcome, such as the development of methods that can facilitate nuclease delivery or expression to only diseased cells or tissues, and the development of new strategies that can enhance HDR in disease-associated postmitotic cells in vivo.

8.4 Limitations and potential obstacles

In the design and implementation of clinical trials, the demonstration of clinical need and pre-clinical supportive data may actual be the least of the

potential challenges. In rare diseases such as RDEB, one of the critical aspects is patient recruitment. Based on my experiences within the field, this is particularly applicable to the recruitment of adult patients. Within the UK there are only four tertiary centres with multidisciplinary teams dedicated to the care of adult patients with RDEB: Guys and St Thomas' NHS Trust in London, Birmingham, Solihull and Edinburgh. Recruitment for studies requiring 5 or more patients may require involvement of multiple centres, and therefore requiring multiple Site Specific R+D applications. Identification of patients most suited for enrolment may also be problematic as in a number of cases mutation analysis and baseline skin biopsy specimens have either not been performed or had been archived. Access to patient data outside of GSTT would be prohibited and only available to those holding honorary contracts within each trust. Another challenge I had come across during patient's selection studies has been patient engagement with clinical trials, and those involving gene therapy clinical trials in particular. Anecdotally, gene therapy was either poorly understood or terrifying for the individual and family. Cell therapies such as allogeneic fibroblast or MSC therapies were easier to conceptualise, and discussion of the theoretical unknown oncogenic potential of gene therapy to a cohort of patients with marked anxiety regarding the development of SCC was challenging. These experiences led to my desire to explore these opinions objectively and hence I conducted the survey detailed in Chapter 5. Interestingly over half of those questioned in the survey would consider taking part in a gene therapy clinical trial in the future and similar numbers would consider intradermal injections as well as a genetically corrected graft. This was wholly unexpected, in particular as these patients

have a chronic, debilitating disease which is characterised by hours of painful dressing changes required on a daily basis. One would assume that performing painful invasive procedures such as intradermal injections into chronic wounds or skin grafts would be intolerable. The unexpected enthusiasm in over 50% pf patients for these trials indicates the level of desperation and desire for a possible cure.

Despite these findings, recruitment adult RDEB patients into clinical cell and gene therapy clinical trials using GSTT as the sole centre is challenging. Patients with RDEB have multiple medical issues and are often chronically sick. Adult patients also have far more complicated social issues that may limit involvement in clinical trials. The majority of the patients recruited to the EBGen study also live outside of London, and therefore the necessity for each study visit needs to be carefully considered, and where possible study visits need to be tied in with routine clinical care. Options for data collection via patient reported outcomes via electronic patient diaries should be used.

There are also challenges within such a small cohort of patients as there may be more than one clinical trial occurring concurrently, with both the patient and researcher required to determine the most suitable patient for each study.

The challenges faced in recruitment of adult patients has been in stark contrast to my experience within the EBSTEM study performed in a paediatric population, in which families were highly motivated and driven to take part. It could be argued that trials in children, before the irreversible contractures and scarring had occurred, should be the main focus for future studies.

Furthermore, the exclusion of patients with a previous squamous cell carcinoma depletes numbers of potential adult participants significantly. During the last year alone, seven diagnoses of SCC occurring in patients with no previous malignancy has been noted at GSTT. In my view the necessity for exclusion of these patients in phase 1 studies of gene therapy is still important. Perhaps the results of initial studies and assessments of off-target genomic analysis at graft sites would provide sufficient safety data to inclue those with a previous history of SCC, the difficulty then being for the clinician to identify suitable areas with no evidence of dysplasia for harvesting skin biopsies and subsequent grafting.

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APPENDIX

Appendix 1. Results of open-ended questions from RDEB patient survey

QUESTION	RESPONSE
BASED ON YOUR CLINICAL TRIAL EXPERIENCE, COULD YOU DEFINE WHAT IS A POSITIVE OR NEGATIVE CLINICAL TRIAL EXPERIENCE?	"All I can remember is I've never had any result that actually helped me for future" "Positive: The researcher explaining the process clearly, but in line with the persons' intellect; don't spea down to anyone, but don't use language that only fellow scientists would understand. Build a rapport. If the will be pain as part of the trial, the patient needs to feel safe with you, and creating even a basic friendshi will help that. When the treatment is being given, allow the patient to react as they need to. Don't laugh o scold, unless they are aiming curse words etc at you. Don't rush through administering the treatmen particularly if it involves pain. Allow time for the patient to take a breath, and ready themselves again. Kee them up to date on results from the trial. That is the experience I had, and it was wholly positive. A negativ experience would simply be the opposite of the above to me. "Positive in the sense one always hopes that the trials will be of benefit to sufferers, whether that be m personally or others in the future" "positive would be constant contact throughout and for one month after it finishes to check if your still oka drs thatexplain everything to you and perhaps get to know you a bit before the trial starts because trust important"

	“Wound healing is great but pain is unbearable while the injection. Although worth it would do it again.”
CAN YOU EXPLAIN YOUR ANSWER (REGARDING PARTICIPATING IN GENE THERAPY CLINICAL TRIAL”AND GIVE REASONS WHY YOU WOULD OR WOULD NOT TAKE PART ?	“I have had a lot skin grafts since i was a young child as i have had many hand surgery's. they took the ski graft from my thighs. as for genetically corrected cells, i have never had and to be honest i'm unsure of havin that as i don't know much about it or have much experience with it”
	“I have wounds that are so painful, they directly inhibit my ability to live the best life I can. The life I used t have. Also, our researchers have worked so hard to reach this type of gene therapy, it would be folly to wa treatment, but refuse to try them. Im more than happy to help find out if this would work, and how well.”
	“I would consider it as I want to try to heal myself and hopefully others with rdeb”
	“…I am keen and willing to support any initiatives that may be of benefit, be that for me 'today' or others the future.”
	“I know it's not a cure yet but if one piece of skin is corrected maybe with more trials more of my affecte skin can be corrected”
	“Having had my hand operated on and a skin graft done on my leg to provide the skin for my hand, I can se the negative effects the procedure can create on the donor site and also issues that can arise with the newl placed skin.”
	“It seems more invasive than the previous option I would like to know more about what this goes to achiev if it's long-term”
	“I have already had a biopsy taken for this”

Appendix 2. RDEB patient survey on gene therapy

This survey is about gene therapies for the condition Recessive Dystrophic Epidermolysis Bullosa. Considerable progress is being made in the search for effective treatments for EB, with early stage clinical trials in progress or planned for several therapies. Our department would like to find out about patients' own understanding of gene therapy and willingness to take part in future related clinical trials and studies.

The results will help clinical researchers design better clinical trials for RDEB that will hopefully lead to new and more effective treatments.

The results of the survey will be confidential and anonymous. Your responses will not affect your regular clinical care.

The survey should take no more than 10 minutes to complete.

First a little bit about you...

1.How old are you?

a) 16-30

b) 31-50

c) > 50

2.Are you male or female – please indicate below?

a) Male

b) Female

3. Have you been diagnosed with Recessive Dystrophic Epidermolysis Bullosa?

a) Yes

b) No

4. Have you ever taken part in a clinical trial?

a) Yes

b) No

If Yes do you have any comments on your experience with the clinical trial, both positive and negative (Free text)

4. Have you heard of a new treatment for RDEB called "gene therapy"?

a) Yes

b) No

c) I have heard of gene therapy but not for RDEB

5. Gene therapy is the correction of a disease by genetic modification of a person's own cells. This would be introducing a 'good' gene into the patient's cells to treat the disease, but it won't change the chance that the disease will be passed on to the patient's children. A patients' own skin cells would be first collected via a skin biopsy* and grown in a lab. After replacement with the missing gene, the skin cells can then be given back to the patient. There are a few methods of giving these cells back to the patient, such as an injection into the skin or even growing the skin cells into a sheet of skin which could be placed directly over a wound.

(*) A skin biopsy is a procedure taking a small piece of skin the size of a 5p piece. This is done under a local anaesthetic so that the area is numbed beforehand. One stitch is usually all that is required to close the wound.

6. Would you consider having a skin graft* of genetically corrected cells?

(*) A skin graft is a piece of skin usually measuring 5 x 5 cm which can be attached to a wounded area either via glue or with stitches around the edges.

a) Yes

b) No

c) Maybe

If not then why not?

Free text

7.Would you be willing to have a skin graft on an area of your body that was unblistered?

a) Yes

b) No

c) Maybe

8.Would you be willing to have a skin graft of an area of your body that was recently blistered?

a) Yes

b) No

c) Maybe

9.Would you be willing to have a skin graft of an area of your body that was chronically wounded ie a wound that had been present for more than 6months?

a) Yes

b) No

c) Maybe

10.Would you consider having an injection (*) of genetically corrected cells into a wound?

(*) An intradermal injection is given via a small needle into the skin.

a) Yes

b) No

c) Maybe

11. If not then why not?

c) Maybe

12. Currently most planned clinical trials using gene therapy are able to treat localised areas rather than the whole body at once. Is there a specific wound or area that you would be keen to have treated first?

a) Yes

b) No

If yes please list the areas and order of preference (top 3)

13. As RDEB is a rare disease, many research centres are now collaborating in order to maximise the potential of treating patients. Would you be willing to travel abroad to receive a treatment such as an injection of cells into the skin or a skin graft?

a) Yes
b) No

If so would you be willing to travel outside of the EU?

a) Yes
b) No

Thank you very much for participating!

Appendix 3. Birmingham Epidermolysis Severity Score

Birmingham EB Severity Score Sheet (Adult)

Patient's name.....DOB.....Type of EB.....

Scorer's name.....Date.....

**See overleaf for detailed instructions*

Score item	Measure	Max	Actual score
*Nails	Lost nails = 4 Dystrophic nails = 8	5	
*Area	½ x % damaged skin: blisters,erosions,scabs, healing skin,erythema,atrophic scarring; not dyspigmentation, or well-healed scars.	50	
*Mouth	0 = no mucosal involvement 1 = occasional blisters/erosions 2 = frequent blisters 3 = persistent symptoms, early structural abnormality 4 = Moderate structural abnormality 5 = severe structural abnormality (see over for detailed scoring for each site)	5	
*Eyes		5	
*Larynx		5	
*Esophagus		5	
Scarring of hands	0 = no scarring 1 = Milia and atrophic scars 2 = Just detectable contractures or webbing 3 = obvious contractures, or proximal webbing 4 = Between 3 and 5 5 = Mitten formation with fingers all fused	5	
Skin Cancer (SCC)	Number of skin cancers +1 for local/regional/lymph node spread +2 for distant metastatic spread, up to maximum score 5	5	
Chronic wounds present for > 6/12	0 = none 1 = <1% body surface area (1% = palm size) 2 = 1-2% 3 = 2-5% 4 = 5-10% 5 = >10%	5	
Alopecia due to EB	0 = no alopecia from EB 1 = 1-19% scalp involvement 2 = 20-39% 3 = 40-59%, 4 = 60-79% 5 = 80-100%	5	
Nutritional compromise	0-5 (where 0 = normal and 5 = cachetic)	5	
TOTAL SCORE		100	

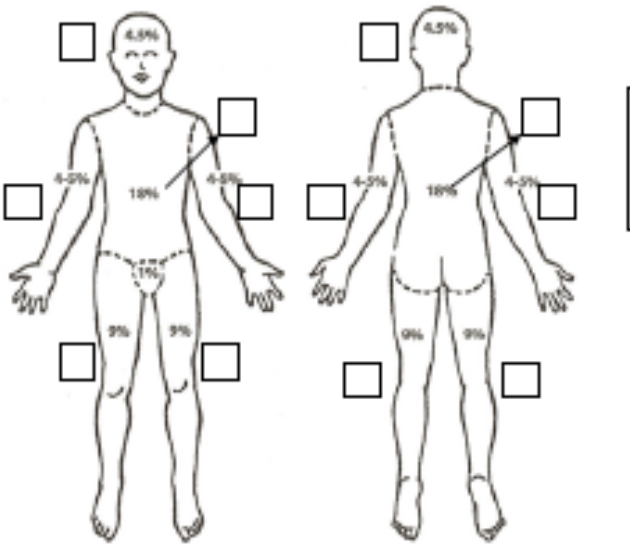
How to fill in the BEBS score sheet

Nails: enter number in each box and add up horizontally

	R hand	L hand	R foot	L foot	Subtotals A	Subtotals B	Total score
lost nails	+	+	+	+	=	+ 4 =	} =
dystrophic nails	+	+	+	+	=	+ 8 =	
normal nails							
total	5	5	5	5			

Area:

Please shade in affected areas on the diagram, then work out percentage for each part and fill in the numbers in the adjacent boxes.
eg if half of the anterior trunk is affected, then put 9% in the box on anterior trunk.
Patient's palm size area corresponds to 1% of total body surface area



Front total = %
Back total = %
Front + back = %

÷ 2 = Area Score =

Mouth, Eyes, Larynx, Esophagus: detailed scoring

	Mouth	Eyes	Larynx	Esophagus
0	No problem from EB	No problem from EB	No problem from EB	No problem from EB
1	Occasional soreness	Occasional soreness	Occasional hoarseness	Occasional dysphagia
2	Frequent soreness	Frequent soreness	Frequent hoarseness	Frequent dysphagia
3	Persistent soreness Just detectable tongue tethering	Persistent soreness early visible external eye disease	Persistent hoarseness	Persistent dysphagia
4	Between 3-5	Between 3-5	Between 3-5	Between 3-5
5	Severe tongue tethering & microstomia	Bilateral sight-threatening eye disease	Life threatening laryngeal obstruction	Difficulty swallowing solids & liquid